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Clinical applications of DNA methylation in gastrointestinal cancer

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Clinical applications of DNA methylation in gastrointestinal cancer

Proefschrift
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door **Michiel Frank Gerard de Maat**

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“Hoe ver je gaat heeft met afstand niets te maken, hooguit met de tijd.
Hoe diep je gaat heeft met denken niets te maken, hooguit met een wil.
Hoe recht je staat heeft met zwaarte niets te maken, hooguit met de wind.”

vrij naar: omarm me, Bløf

Aan mijn ouders,
Voor Debbie.

Cover:

Digital impressionistic collage of sodium metabisulfite crystal formation in aqueous solution. Treatment of DNA with sodium metabisulfite is the key step to detect whether DNA is methylated.

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General introduction



Gastrointestinal cancers

The lineage of the lumen of the gastrointestinal (GI) tract from mouth to anus consists of mucosal cells that separate the inner world of the body from the outer environment. The functions of these cells are numerous as well as diverse: active uptake of nutritional elements, passive passage of fluids and electrolytes, secretion of digestive enzymes, defense from pathogenic bacteria, facilitating stool transportation etcetera. To enhance its performance and exchange capacity the mucosal surface is increased by extensive folding on the macroscopic level: i.e. plicae gastricae of the stomach, Kerckring's folds in the small bowel, haustrae of the large bowel and on the microscopic level: i.e. gastric foveolae and colonic crypts and villi. If spread out, the total surface of the digestive tract would be the size of approximately two tennis courts. The regenerating capacity of the GI tract lineage is tremendous; i.e. the mucosal layer of the large intestine completely renews itself every 5 days. The intensive interaction with outer environment and the high rate of cell division may contribute to the fact that cancers of the gastrointestinal tract are among the most widely seen in humans. In 2008 in the United States, 271.290 new cases of GI cancer were diagnosed accounting for 19% of all cancers and GI cancers caused 24% of all cancers deaths (see **table 1**)¹.

Cancer Type	Netherlands		United States	
	New cases (n in 2000)	Number of deaths (n in 2000)	New cases (n in 2000)	Number of deaths (n in 2000)
Stomach	1938 (2134)	1450 (1719)	21500 (21500)	10880 (13000)
Colorectal	11231 (9236)	4709 (4274)	148810 (130200)	49960 (56300)

Table 1: GI cancer incidence and mortality rates in the Netherlands and United States in 2008.

Causative genetic mutations have been identified in some hereditary forms of GI cancers such as Lynch syndrome²⁻⁵ (former hereditary non-polyposis colorectal carcinoma, HNPCC) and familial adenomatous polyposis (FAP)⁶. The exact cause of sporadic cancers of the GI tract is unknown. Suggested contributive factors in causing gastrointestinal cancers are; dietary-related (red meat intake⁷, folic acid⁸, fiber intake^{9, 10}), alcohol and smoking^{11, 12}. Another associated factor is obvious but often overlooked and associated with the vast majority of solid tumors; age. Some macroscopic and microscopic precursor lesions of GI malignancies are known. In colorectal cancer sessile, serrated or hyperplastic polyps¹³ or aberrant crypt foci¹⁴ are recognized premalignant abnormalities and in gastric cancer intestinal metaplasia or foveolar hyperproliferation¹⁵⁻¹⁷. In premalignant lesions, cell division activity is disturbed, however, the cells remain within their histological architectural matrix. At some point, and this is the key step, the cells gain the capacity to invade through the basal membrane of the mucosa which defines the adenocarcinoma diagnosis. In the submucosal layer the cells can enter the lymphatic system or the bloodstream causing distant spread to lymph nodes or other organ sites causing GI cancer mortality.

This thesis aims at the two organs of the digestive system that are most often hit by malignant neoplasia: The stomach and the large bowel. Although over the last decades there has been an increase in the incidence of esophageal¹⁸, gastric cardia¹⁹ and pancreatic cancer²⁰ this ranking remains unchanged over the last 20 years.

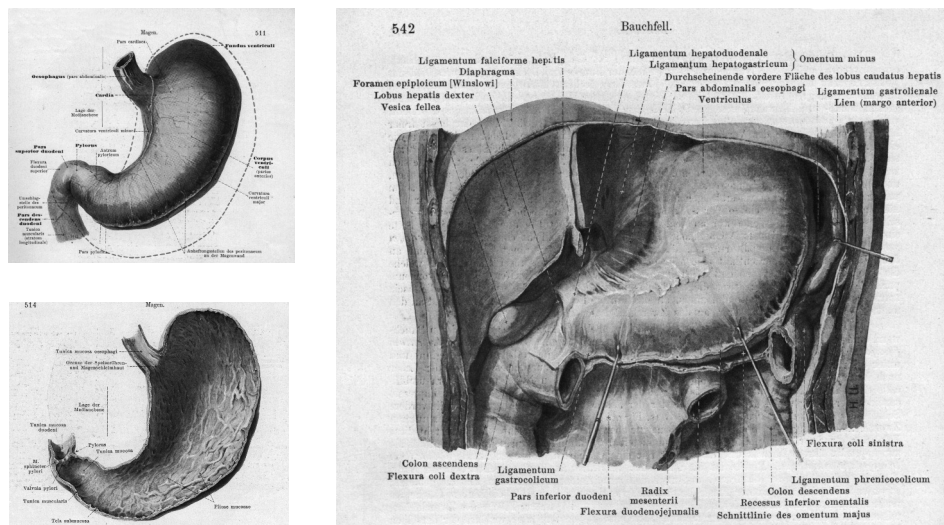


Figure 1: Anatomy of the stomach and its position in the abdomen.

Source: Handatlas der Anatomie des Menschen, Dritter Band, 1909.

Gastric Cancer

Worldwide, neoplasms of the stomach (**figure 1**) are second in causing cancer mortality¹. Gastric cancer compared with other GI cancers is known for its geographically various incidence and mortality²⁰. In Asian countries such as China, Korea and Japan gastric cancer has the highest incidence and mortality of all malignancies. In Western countries approximately 50 years ago gastric cancer was also the most predominant cancer, although incidence rates dropped tremendously over the last decennia. In Portugal, however, gastric cancer incidence and mortality is highest and this is exceptional compared with other European countries²¹. Dietary factors such as high intake of salted and smoked foods may explain this geographical variation. Increasing consumption of fresh ingredients in the wealthier Western world may account for the decline of gastric cancer incidence. Also the various incidences of gastric infections with *Helicobacter Pylori* bacteria or *Epstein-Barr* virus which are considered precursor stages of gastric cancer may contribute. Clinical symptoms are non-specific and mimic common diseases like dyspepsia or ulcer disease. Weight-loss or passage problems are late symptoms and the disease often is incurable at this stage. Five-year survival rates of gastric cancer in the Western world are deplorable (10-20%) and despite many efforts this has not markedly improved over the last years²². Scientific interest for gastric cancer in Western countries has faded as incidence rates drop. Gastric cancer's relative unresponsiveness to the common systemic chemotherapeutics also contributes to this²³.

The mainstay of gastric cancer treatment is radical surgical removal of the complete or partial stomach with its surrounding lymph node stations. The extend of lymph node removal has been a subject of study and more extensive nodal dissection (including those surrounding the great vessels) may improve long-term disease survival rates, however, induces relatively high perioperative mortality in a Western study population²⁴⁻²⁶. Recently, clinical trials have successfully shown the benefit of neoadjuvant therapy over surgery alone²⁷⁻²⁹. These important studies now have generated more scientific interest to improve gastric cancer treatment. In the Netherlands the CRITICS trial is being conducted that aims to evaluate whether postoperative chemo/radiation therapy in addition to preoperative chemotherapy further improves survival rates^{30, 31}.

Colorectal Cancer

The incidence of colorectal cancer in the Netherlands has increased with an average of one percent per year from 1989 to 2006 (www.ikcnet.nl) and forms a major burden on health care costs^{32, 33}. It can be concluded that the treatment of colorectal cancer is improving as despite the increasing incidence, mortality rates have dropped³⁴. The large bowel appears anatomically and histologically as a continuous tube that is separated from the small bowel by Bauhin's valve and ends at the dentate line where the squamous cells of the anal canal take over from the bowel mucosa epithelial cells. The last 15-20 centimeters distal of the promontorium or the sigmoid fold is called the rectum and this part of the colonic tube is fixed in the smaller pelvis between the bladder and the sacrum. The surgical technique for rectum resection is different from colon surgery due to this close interaction with the anatomical structures within the pelvic cavity. The fixation of the rectum provides opportunities for external beam radiation therapy. In literature large bowel cancer is often summarized as colorectal cancer while, as pointed out above, from a clinical point of view this should be separated into colon and rectal cancer.

Colon Cancer

From the cecum throughout the sigmoid the large bowel lies in the abdominal cavity and is hung up in the great omentum in the hepatic flexure and splenic flexure (**figure 2**). Primary tumor removal can be achieved relatively simple in case there is no invasiveness into surrounding structures (T-stage 1-3). An important indicator of adequate surgery is to harvest sufficient amounts of lymph nodes for an adequate, complete pathological diagnostic procedure (pTNM-staging). Since nodal status is the most important clinical parameter to date much effort is put into optimizing nodal harvest and optimizing evaluation of the retrieved lymph nodes^{35, 36}. Patients with nodal disease spread have shorter survival chances and have been proven to benefit from adjuvant chemotherapy³⁷. Different administration forms and schedules of the current standard FOLFOX-4 (5-FU, leucovorin, oxaliplatin) adjuvant treatment regimen are being studied to have added benefit. Addition of target drugs that specifically inhibit epidermal growth factor receptors (Cetuximab) or vascular endothelial growth factors inhibiting angiogenesis (Bevacuzimab or Avastin®) is under investigation³⁸⁻⁴⁰. Yet 30 percent of patients without evidence of nodal spread will develop distant metastasis and this indicates the need for a better classification of this malignancy by prognostic markers.

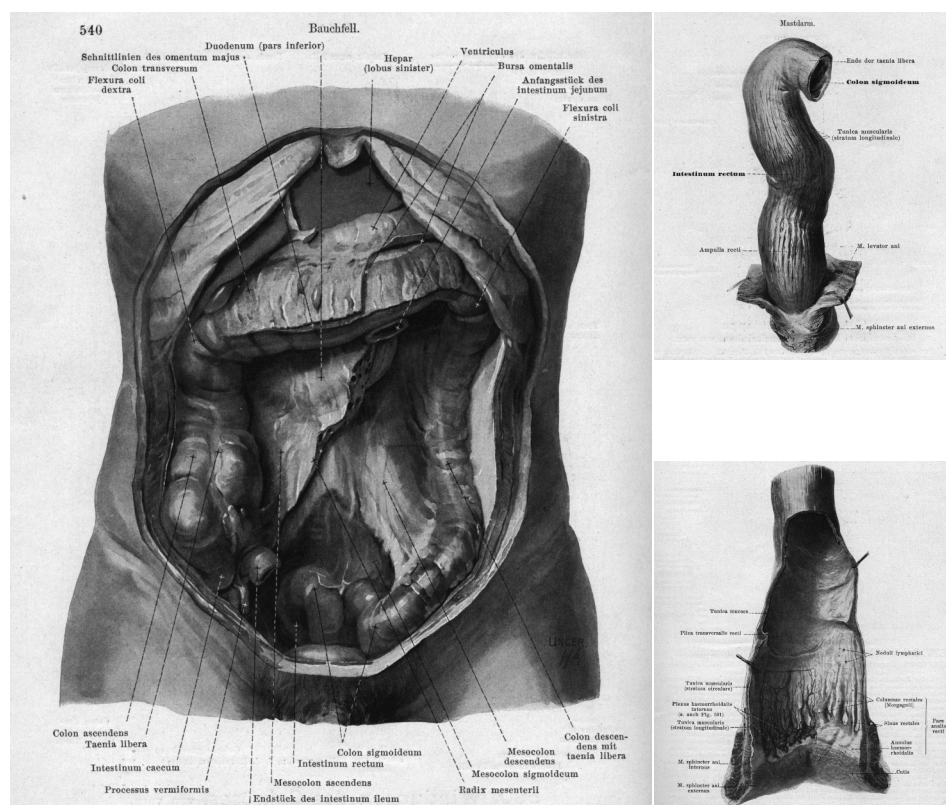


Figure 2. Anatomy of the colon and rectum and its position in the abdomen.

Source: *Handatlas der Anatomie des Menschen, Dritter Band, 1909.*

Rectal Cancer

About 25% of adenocarcinoma in the 150 centimeters long large bowel occurs in the last 15 centimeters of the rectum (**figure 2**). The surgical treatment of rectal cancer, as indicated, is more challenging compared to colon cancer and can be complicated by specific morbidity. For instance accidental laceration of nerves of the sacral plexus can affect urinary and fecal continence and in males erectile function. To better protect the nerves and reduce perioperative blood loss Heald et al. in 1979 described total mesorectal excision (TME) meaning sharp dissection of the so-called avascular mesorectal fascia along with the rectum⁴¹. TME has majorly reduced complications and local recurrence rates of rectal cancer⁴². Many clinical trials have been conducted to further reduce local recurrence rates that test preoperative or postoperative strategies⁴³⁻⁴⁵. Benefit of adjuvant therapy to reduce distant recurrence rates is currently under investigation in the SCRIPT trial (www.dccg.nl/trials/script). There are specific prognostic parameters in rectal cancer such as involvement of the circumferential margin^{46, 47} and distance from the anal verge⁴⁸ and these specific parameters should be included in studies evaluating rectal cancer.

No. 4356 April 25, 1953

NATURE

737

MOLECULAR STRUCTURE OF NUCLEIC ACIDS

A Structure for Deoxyribose Nucleic Acid

WE wish to suggest a structure for the salt of deoxyribose nucleic acid (D.N.A.). This structure has novel features which are of considerable biological interest.

Figure 3: detail from the original publication presenting the helical structure of DNA by Watson and Crick and in Nature journal from 1953

Molecular Oncology

The significance of the discovery of the molecular structure of DNA for clinical medicine has been gradually appreciated among physicians since 1953⁴⁹⁻⁵¹. Molecular biology is a basic element of medical training nowadays. Medical students are taught the fundamental principles of DNA replication, its transcriptional regulation, the mechanism of protein synthesis and are made familiar with techniques for DNA analysis. Over the next years, molecular medicine will become increasingly integrated into daily medical practice. Oncology is an important field of research for molecular scientist where many new discoveries on disease mechanisms at the molecular level are being made. Cancer research and molecular oncology in particular will continue to be a field of invention as this disease still puzzles us and merits a larger portion of our scientific efforts.

Over the last years it has become increasingly clear that genetic alterations majorly contribute to the development of gastrointestinal adenocarcinoma. The nature (mutations, amplifications, translocations etc.) of genomic instability of GI cancer is being unraveled fast after the completion of the human genome project (www.genome.gov) and development of high resolution techniques that can analyze a individual's complete genome in only a few weeks. On the DNA level, cancer cells differ greatly from healthy cells. Early in tumorigenesis genomic stability is affected and results into gene alterations that are key molecular pathogenic steps⁵². A current hypothesis is that by acquiring a sufficient number of alterations in tumor suppressor genes and oncogenes a normal bowel mucosa epithelial cell will transform and promote tumor progression^{53, 54}. Identification of the key molecular changes of tumorigenesis will allow us to get a grasp on the malignant process and will offer targets for treatment. As our knowledge of cancer cell molecular biology expands, our models of disease mechanisms expand and certain dogmatic hypothesis need updating. Molecular medicine is an evolving field that is gaining importance in daily medical practice especially for those specialists involved in the treatment of cancer patients.

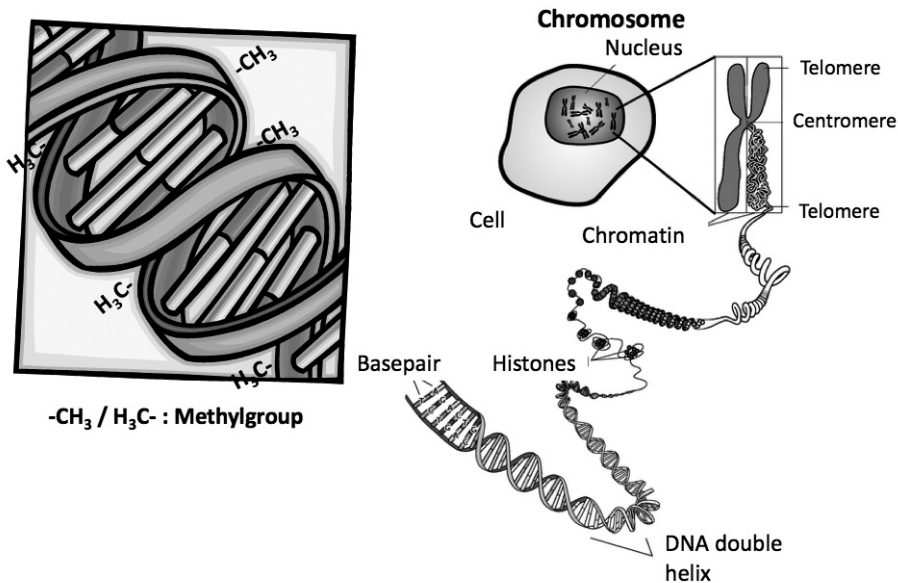


Figure 4: Representation of DNA unraveling, showing the level at which two important epigenetic regulatory mechanisms (histon proteins, methyl group placement) take place.

Genetics versus Epigenetics

Alterations in the human genome involving the sequence of the four components of DNA, the nucleic acids (cytosine, guanine, adenine and thymine), are part of the field of genetics. Next to the nucleotides that form the double helix (Figure 4) there are many elements that are crucial for the main function of the human DNA, the process of protein transcription. These factors, i.e. methyl groups, histone proteins and their tails, chromatin structure remodeler complexes (**figure 4**), are part of the world of epigenetics^{55, 56}. The current definition of epigenetics is as follows: All heritable traits (over rounds of cell division, even transgenerationally) that do not involve the DNA nucleotide sequence. This new field is being more and more appreciated and also plays a key role in carcinogenesis as genetics does^{57, 58}. As genetics is becoming part of our oncology world, epigenetic factors controlling transcription inevitably comes with it. This dissertation aims at one specific epigenetic factor namely, DNA methylation.

DNA methylation

The nucleus of each cell in the human body contains the same identical copy of genomic DNA. Differentiated cells from different organ structures perform specific functions requiring synthesis of specific proteins. Each cell, however, has the necessary information to produce all proteins encoded by the human genome. One can imagine that most cells have no need for the whole range of proteins. A large bowel mucosal cell for instance has no need for genes that encode proteins to form striated muscle. Synthesis of some proteins could disturb a cell's homeostasis when produced. How embryonic cells develop into differentiated cells and how differentiated cells suppress transcription of unwanted or unnecessary proteins is an important question in cellular biology.

In 1948 it was first observed that a methyl-group was placed on the fifth position of the cytosine nucleotide (m5Cyt) in calf thymus DNA⁵⁹, 5 years before the molecular formation structure of DNA was established. Since then it has taken a dedicated group of scientists several decades to unravel what role this small methylgroup (CH₃) has in molecular cell biology. From the start, the hypothesis was postulated that cytosine methylation had a role in transcriptional regulation, however, there was no experimental evidence to support this⁶⁰. Waalwijk et al.'s classic experiments showed differential methylation patterns between various organ tissues in rabbits which was highly suggestive for the role of methylation in cellular differentiation⁶¹. A role in differentiation was likely, as it was known that specific methylation patterns exist; that methylation was symmetrical in both DNA strands and that methylation patterns are clonally heritable⁶⁰. Other hypothesized functions of methylated DNA were that it would protect DNA from being cut by eukaryotic restriction enzymes. It also may play a role in DNA replication as was known that replication stops when DNA is unmethylated⁶². A protective role was suggested as it was observed that unmethylated DNA is more prone to spontaneous mutagenesis^{63, 64}. Also it was found that methylated sequences are more abundant in centromeric regions of a chromosome that indicates a role in chromosome structure and possibly folding⁶⁵.

Riggs et al. proposed in 1975 a model to explain the initiation and maintenance of mammalian X chromosome inactivation and certain aspects of other permanent events in eukaryotic cell differentiation⁶⁶. A key feature of the model is the proposal of sequence-specific DNA methylases that methylate unmethylated sites with great difficulty but easily methylate half-methylated sites. Peter Jones et al. in the late seventies made a key observation when he incubated mouse fibroblast-like embryonic cell lines with 5-azacytidine, a chemotherapeutic agent that was tested for treating leukemia⁶⁷. He noticed after several rounds of replication that the cells could be characterized as contractile striated muscle cells, differentiated adipocytes and chondrocytes capable of the biosynthesis of cartilage-specific proteins⁶⁸. He realized that this chemical must have activated genes that were silenced. Later on it was demonstrated that 5-aza was a specific inhibitor of DNA methyltransferase (*DNMT*) enzymes that are responsible for transferring methylation patterns from mother to daughter cell during replication⁶⁹⁻⁷¹.

Chemically, due to the open groove in the double helix, placement of a methyl-group is only possible when cytosine is followed by guanine. An important observation was that the CpG dinucleotide doublet is found less frequently in the human genome than is statistically expected⁷². These doublets are more concentrated in coding regions compared with non-coding regions and this suggests they have a role in transcription. During the completion of “the human genome project” it could be more definitely supported that CpG dinucleotides are located predominantly in coding regions. More specifically, they are found in high concentration in gene promoter regions along with signaling molecules recognition sites (TATA boxes, helicase binding sites etc.) and those sites were named CpG islands⁷³. About 65% of all human genes have promoter regions that coincide with CpG islands⁷⁴. It is generally accepted that the human genome was more CpG rich earlier in evolution and that over time due to the earlier mentioned susceptibility of CG dinucleotides to spontaneous mutation/reduction to CT, only the CG’s protected by a methyl-group were conserved. Heavy methylation or hypermethylation of gene’s promoter region is associated with under-expression of that gene (see **figure 5**)^{60, 75}. This is likely due to the sterical inaccessibility of the promoter region. In short, the human genome contains CpG dinucleotide rich islands that coincide with gene promoter regions that are methylated or can undergo de novo methylation which results into silencing of that gene.

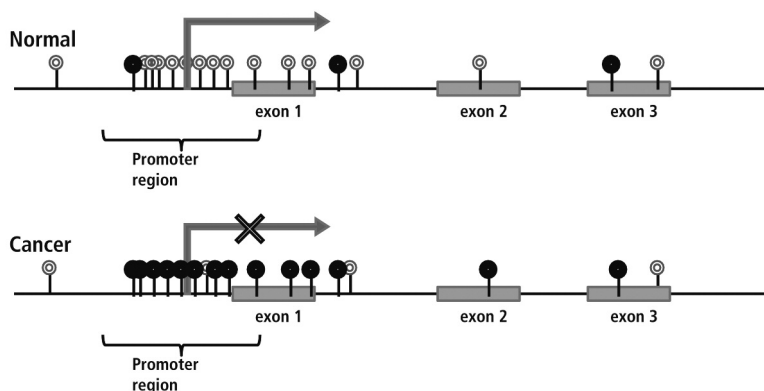


Figure 5: Representation of gene silencing by methylation. Boxed areas indicate gene exons.

● are methylated CpG dinucleotides, ○ are unmethylated CpG sites.

The arrows indicates the start site of transcription.

DNA methylation and cancer

Feinberg and Vogelstein were the first to test whether differences exist in DNA methylation between cancerous and non-cancerous cells of the same patient⁷⁶. They found that DNA of a variety of different human cancers was hypomethylated. They could show activation of some important oncogenes by hypomethylation, i.e. RAS⁷⁷, MAGE⁷⁸ and CT⁷⁹. Subsequently, many genes thought to be important in cancer were studied for methylation status and surprisingly some genes were also found to be densely methylated (see **table 2**)

and this was first observed in calcitonin⁸⁰. Later on, retinoblastoma (Rb), the first tumor suppressor gene, was found to be silenced by hypermethylation in cancer^{81, 82}. A current hypothesis adds silencing of tumor related genes by DNA hypermethylation as another form of a second hit to Knudson's hypothesis (see **figure 6**)⁸³. One must look at methylation in cancer as an example of epigenetic dysregulation, with both hypomethylation and hypermethylation having significant roles.

Gene abbreviation	Gene name	Location	Gene function
APC	Adenomatous polyposis coli	5q21	Signal transduction
BRCA1	Breast cancer 1	17q2	DNA repair
DAPK	Death associated protein kinase	9q34	Evasion of programmed cell death
CDH1	E-cadherin	16q22.1	Cell adhesion
ER	Estrogen receptor	6q25.1	DNA binding, transcription activation
GSTP1	Glutathione S-transferase P1	11q13	Cell cycle regulation
hMLH1	Human monologue of MutL in bacteria	3p21.3	DNA mismatch repair
MGMT	O-6- methylguanine-DNA methyltransferase	10q26	DNA repair
p14 (ARF/CDKN2a)	Cyclin dependent kinase inhibitor 2a	9p21	Cell cycle regulation
p15 (INK4b/CDKN2b)	Cyclin dependent kinase inhibitor 2b	9p21	Cell cycle regulation
p16 (INK4a/CDKN2a)	Cyclin dependent kinase inhibitor 2a	9p21	Cell cycle regulation
RARb2	Retinoic acid receptor beta 2	3p24	DNA binding
SOCS	Suppressor of cytokine signaling	17q25.3	Suppression of cytokine signaling
TIMP3	Tissue inhibitor of metalloproteinase 3	22q12	Tissue invasion and metastasis

Table 2: Important tumor-related genes known to be silenced by promoter hypermethylation in solid tumors (source: Bernal et al., *Biol Res*41: 303-315, 2008)

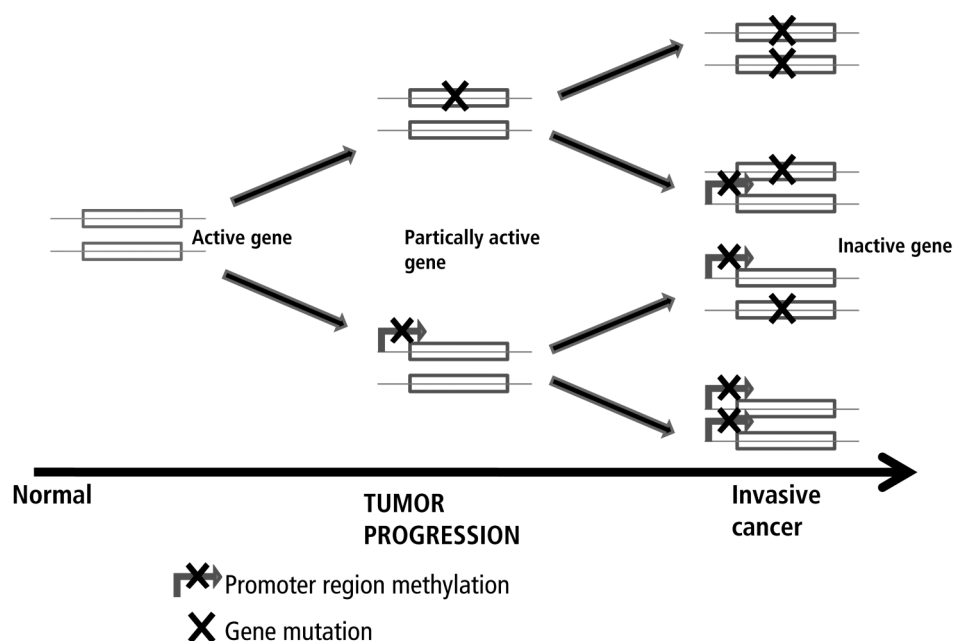


Figure 6: Adjustment of Knudson's hypothesis on gene inactivation

Analytical Techniques

The unraveling of the role of DNA methylation in human biology and oncology has been hampered by technical limitations. Initially, chromatographic- (gas, liquid) or mass-spectrometry methods were used. The discovery of methylation-specific restriction enzymes was a major step forward, but analysis of methylation status of DNA sequences was limited to the recognition sites of the known enzymes. A major milestone in molecular research in general was the discovery of the protocol for polymerase chain reaction (PCR) by Kary B. Mullis described first in 1986⁸⁴⁻⁸⁶. Any sequence of the human genome could now be amplified, with high specificity and reliability to a great amount of template majorly allowing rapid cloning and analysis of DNA. An important improvement in methylation research was the introduction of sodium metabisulfite treatment of DNA described by Suzanne Clark et al. first in 1994⁸⁷. The chemical instability of cytosine nucleotides and their proneness to deamination when unmethylated was utilized. DNA was incubated with a mild reductor (sodium metabisulfite) and showed specific conversion of cytosine nucleotides to thymine but not when methylated. Followed by direct sequencing analysis it can be detected if the DNA was methylated or not. Herman et al. then introduced DNA bisulfite treatment followed by PCR amplification with primers specific for methylated or unmethylated sequences⁸⁸. This protocol was highly sensitive and applicable in many laboratory settings and most importantly required only small amounts of sample DNA. When this thesis' studies were initiated the most widely used technique for DNA methylation assessment was DNA bisulfite modification followed by methylation specific PCR (MSP) followed by gel electrophoresis of PCR products.

Micro Satellite Instability

In about fifteen percent of all cases of large bowel cancer there is positive family history. The most frequent form of hereditary colorectal cancer is Lynch Syndrome (former HNPCC). On the molecular level this cancer is hallmarked by mutations affecting DNA repair genes and thus impairing the proof reading mechanism after DNA replication in cell division⁸⁹. Mismatch repair (MMR) genes affected by mutations in Lynch Syndrome are MLH1, PMS2, MSH2 and MSH6. MMR deficiency can be detected by comparing the length of non-coding DNA repeat sequences, called microsatellites, of cancer cells with those of healthy cells that become shorter due to the defectiveness to repair these missing nucleotides after cell division^{4, 5, 90, 91}. This so-called microsatellite instability (MSI) is also found in about 15% of colorectal patients without a positive family history and in whom no mutation in MMR genes is detected. Colorectal tumors with sporadic MSI show specific histologic features such as mucin excretion, poor differentiation, lymphocytic infiltration and clinical features such as location in the proximal colon and female sex^{92, 93}. It has now been firmly established that in these patients shutdown of the *hMLH1* mismatch repair gene by promoter region methylation is causative for sporadic MSI^{94, 95}. This interesting interaction between genetics and epigenetics is further looked into in this thesis's studies.

CpG Island Methylator Phenotype

The epigenetic silencing of tumor suppressor genes appealed to many researchers as a key causative mechanism of carcinogenesis and lead to a hunt for densely methylated CpG islands in human cancers. Issa et al. developed a screening method that compared human CRC cell line DNA to that of healthy donor lymphocytes⁹⁶. Differentially methylated sequences were located on the human genome, confirmed to adhere to the CpG island definition and this lead to 33 so-called methylated-in-tumor (MINT) loci. The MINT loci did not relate to any coding sequence or promoter region and some could be verified by methylation specific PCR to be specifically methylated in colorectal tumor tissue^{97, 98}. It was observed that methylation of several MINT loci often occurred simultaneously and in concurrence with methylation of important tumor related genes such *p16* and *hMLH1*. Also, tumors with increased methylation showed MSI and a CpG island Methylator Phenotype (CIMP) colorectal cancer was proposed. Later, CIMP was further established as correlations were shown with *KRAS*, *BRAF* and *p53* mutational status⁹⁹. The phenotype character of CIMP was challenged as this group of tumors could also constitute the far end of a continuous spectrum of methylation in tumor and not a distinct, clearly separable group^{100, 101}. CIMP currently is still not clearly defined and different research groups use different marker-sets and techniques and show different clinical correlations. There are some consistent findings on clinical parameters associated with CIMP+ colorectal tumors: Older age, proximal location in colon, female sex, however, not surprisingly these all match MSI positive CRCs. Whether CIMP will be as significant in CRC as MSI is still under investigation.

Biomarkers

In daily medical practice, biomarkers are highly important tools for physicians in diagnosing diseases, estimating disease severity, follow-up of disease progression, exclusion of pathology etc. Next to diagnostics, markers are also used to help clinical decisions to start,

stop, adjust, switch or postpone treatment. Some biomarkers are direct reflectors of conditions (i.e. serum hemoglobin levels for anemia, however, indirect, surrogate markers are also frequently used that are reflective of conditions (i.e. C-reactive protein or CRP for infection). In GI cancer, the TNM-staging system is currently the best tool we have for making treatment decisions. There are indications, however, that there is room for improvement. First it is first important to realize that our treatment for GI cancer is empiric and non-specific. Aggressive surgery and chemo- and radiotherapy come with considerable morbidity and mortality. Better subclassification of GI tumors is needed to prevent undertreatment in some cases as well as overtreatment in others. The TNM system only includes surrogate parameters of disease progression. Primary tumor cell features, especially in GI cancers, currently have a limited role in clinical decision making. Molecular biomarkers assessed in the primary GI tumor form a new area of cancer diagnostics and are currently under investigation for their use. The use of epigenetic biomarkers in GI cancers is a relatively new field compared with genetic biomarkers. The single, most important objective of this thesis' studies was to test whether epigenetic biomarkers have potential to subclassify GI cancer patients into clinically relevant groups. The role of this novel category of biomarkers in prediction of gastrointestinal cancer disease outcome was evaluated. Subsequently we continued to assess whether these markers merit further evaluation to make decisions tailoring the various multidisciplinary GI cancer treatment options for individual patients.

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Thesis Outline



Thesis outline

This dissertation is divided into two sections. The first section introduces four studies that were performed to further develop and validate techniques for DNA methylation analysis. The second section shows application of the developed techniques and intends to demonstrate that the relatively new field of epigenetics can be utilized for better subclassification of gastrointestinal malignancies to aid their surgical treatment. The studies demonstrate DNA methylation analysis on primary GI tumor tissue to potentially hold value in clinical decision making. The four chapters of the second section focus on gastric cancer, colorectal cancer and on rectal cancer specifically.

Section One

The **first chapter** introduces a method that allows to control methylation-specific PCR reactions better, a technique widely used in DNA methylation analysis. In the study a protocol is described for synthesis of completely unmethylated whole genomic DNA that can be used as a negative control to include in MSP experiment set-ups. The sample DNA is cheaply and quickly synthesized and quantities of DNA are obtained. The introduced so-called universal unmethylated control (UUC) can be used as a standard negative, unmethylated reference sample for any human gene.

The **second chapter** reports on a protocol that dramatically reduces the number of steps needed to obtain sodium bisulfite modified sample DNA from formalin-fixed paraffin-embedded (FFPE) tissue DNA ready for PCR amplification. This protocol eliminates many purification and washing steps used in classic DNA isolation techniques and classic bisulfite treatment methods. The procedure was named on-slide sodium bisulfate modification (SBM) since the formalin fixed paraffin embedded (FFPE) tissue section on the glass slide is incubated directly into sodium bisulfite solution. The DNA is modified in situ, still being in the nucleus. Performance of DNA conversion efficiency is tested and compared with the classic SBM methods. Application is shown in that on-slide SBM enables methylation-specific PCR (MSP) experiments on small tissue areas (1-2 mm²) that previously were difficult to perform. For instance, direct comparison of specific gene promoter methylation status of tumor cells with adjacent adenoma cells to adjacent normal epithelial cells on the same tissue section is now possible.

The PCR technique used in the first two chapters was semi-quantitative and we wished to combine the on-slide protocol with a fully quantitative PCR technique for better detailed DNA methylation assessment. The **third chapter** introduces Absolute Quantitative Analysis of Methylated Alleles (AQAMA). The experiments show that AQAMA can be combined with on-slide SBM and constitutes a robust assay that enables new opportunities to study cancer progression in a better quantitative detail.

The **fourth chapter** describes a protocol that aims to further improve the detailed analytical capabilities of the in-situ DNA modification concept. We integrated in-situ modification with the existing laser capture microdissection (LCM) system. LCM can select and isolate and pick up individual cells or groups of cells from a paraffin tissue section. The developed so-called “on-cap SBM” technique can compare isolated cells from histologically different tissue areas. The studies show feasibility of performing on-cap SBM combined with AQAMA assessment of the

LINE-1 DNA repeat-sequence. Application is shown that LINE-1 methylation levels of primary tumor tissue can discern presence of lymph node metastasis or distant disease spread.

Section 2

After optimizing our analytical approaches for DNA methylation assessment we formulated translational research questions. Gastro-intestinal primary tumor FFPE tissue was available as patient study material from gastric, colon and rectal cancer. The TNM-staging system accurately predicts disease outcome and is used for most treatment decisions for patients with gastro-intestinal tumors. Further subclassification to assess disease aggressiveness, however, is needed to further improve tailoring (neo)adjuvant treatment to radical surgery. Therefore as a central objective of Section 2 DNA methylation analysis was evaluated for its potential to subgroup GI cancer patients and analyze prognostic .

In gastric cancer protein expression of *cyclo-oxygenase-2 (COX-2)* enzyme has been tested to provide additional prognostic information independent from TNM staging. Methods for protein expression assessment, however, are still difficult to standardize and allow for inter observer variation. In **chapter 5** we looked into whether epigenetic status of the COX-2 gene promoter region in gastric cancer patients controls COX-2 expression and whether it can be utilized as a prognostic marker in gastric cancer. Identification of novel biomarkers with good diagnostic performance qualities for gastric cancer has become increasingly important especially since recent studies are showing effectiveness of chemoradiation regimens in addition to surgery where this was unsuccessful in the past.

The next three chapters test clinical utility of methylated-in-tumor loci (MINT) in rectal and colon cancer. MINT loci have been repeatedly shown to be aberrantly methylated in colorectal and gastric cancer, however, their prognostic utility has not been explored widely to date.

In **chapters 6** and **7** we test quantitative MINT locus methylation for its ability to subclassify rectal large bowel adenocarcinomas specifically and whether they can serve as biomarkers to aid treatment decisions in the multimodality treatment approach of rectal cancer. Clearly separate subgroups of rectal cancer patients based on MINT locus methylation levels could be identified. **Chapter 6** focuses on predictive value on the potential of early rectal cancer to distantly metastasize. **Chapter 7** evaluates the ability of predicting rectal cancer local recurrence probability. The data provide evidence for MINT markers to be of potential help in indicating patients towards specific adjuvant treatment regimens aimed to reduce either local or distant recurrence.

In **chapter 8** we test changes in MINT locus methylation quantitatively during CRC progression using our developed techniques AQAMA and on-slide SBM. We study these methylation changes in relation to microsatellite instability and DNA mismatch repair system sufficiency which is an important hallmark of genomic instability of a subgroup of CRCs. The relation between DNA methylation is used to epigenetically subgroup large bowel cancers and this is analyzed for its value predicting distant metastasis probability.

Section 1: Technical Advances



CHAPTER 1

Synthesis of universal unmethylated control DNA by nested whole genome amplification with Φ 29 DNA polymerase

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Abstract

Optimization of highly sensitive methods to detect methylation of CpG islands in gene promoter regions requires adequate methylated and unmethylated control DNA. Whereas universal methylated control DNA is available, universal unmethylated control (UUC) DNA has not been made because demethylase is not available to remove methyl groups from all methylated cytosines. On the basis that DNA synthesized by DNA polymerase does not contain methylated cytosines, we developed a method to create UUC DNA by nested whole genome amplification (WGA) with Φ 29 DNA polymerase. Contamination of the template genomic DNA in UUC was only 3.1×10^7 , below the detection limit of sensitive methods used for methylation studies such as methylation-specific PCR. Assessment of microsatellite markers demonstrated that even nested Φ 29 WGA achieves highly accurate and homogeneous amplification with very low amounts of genomic DNA as an initial template. The UUC DNA created by nested / 29 WGA is practically very useful for methylation analysis.

Introduction

Cytosines of CpG dinucleotides in DNA of higher order eukaryotes are partially methylated¹, and this modification has important regulatory effects on gene expression, especially when it involves CpG-rich areas (CpG islands) in the promoter region^{2,3}. Epigenetic gene silencing by promoter hypermethylation is as significant as deletions or mutations for inactivation of tumor suppressor genes⁴⁻⁶. Because these events play a significant role in malignant transformation and immortalization of cells, assessment of gene promoter hypermethylation has become important to understand tumor progression. Among the available methods for detecting specific methylation status of genes, methylation-specific PCR (MSP) and its derivatives are currently the most widely used techniques because they have high sensitivity for virtually any block of CpG sites in CpG islands⁷. Because the MSP results are highly dependent on the specificity of primer annealing, the annealing temperature of thermal cycling and other PCR conditions must be optimized carefully with proper methylated and unmethylated control DNA to avoid nonspecific amplification which causes false-positives or false-negatives. However, universal unmethylated control (UUC) DNA is not available whereas universal methylated control (UMC) DNA can be made from normal genomic DNA with a CpG methylase SssI⁸. Therefore, DNA from peripheral blood leukocytes (PBL), sperm, or other tissues is usually utilized as an unmethylated control, depending on the methylation status of the target site. However, it is labor consuming and sometimes very difficult to verify the absence of CpG methylation at the target site of the template DNA used as an unmethylated control. In addition, it is impossible to find an unmethylated control for global methylation analysis because there is no completely unmethylated genome in humans. Therefore, artificially synthesized UUC DNA would be highly valuable in any methylation analyses such as global methylation analysis or assessment of promoter hypermethylation of tumor-related genes in tumors and serum. On the basis that DNA synthesized by DNA polymerase does not contain methylated cytosines, we aimed to create UUC by whole genome amplification (WGA), but the conventional thermal cycling WGA methods were not adequate because they inefficiently amplified GC-rich areas⁹, the targets of methylation studies. Recently, a WGA technique by Φ 29 DNA polymerase, which is from the bacteriophage Φ 29, has been developed^{10,11}. The Φ 29 polymerase continuously amplifies single- or double-stranded circular- or linear-DNA by strong strand displacement activity. Therefore, after an initial heat-melting step, the Φ 29 polymerase does not require further thermocycling to initiate nascent strand synthesis and can amplify highly GC-rich sequences. In addition, Φ 29WGA has been shown to have high fidelity and near complete genome representation¹². However, because the amplification power of this method is only 10^3 – 10^4 , we designed a protocol using nested Φ 29 WGA to make a UUC and confirmed its utility for practical methylation studies.

Materials and Methods

Template genomic DNA

The genomic DNA of PBL obtained from healthy donor volunteers was used as the template DNA for WGA. Peripheral blood was centrifuged and the PBL fraction was isolated. DNA was extracted using DNAzol reagent (Molecular Research Center, Cincinnati, OH) and quantified with an UV absorption spectrophotometer.

Creation of UUC by nested WGA with Φ 29 DNA polymerase

GenomiPhi DNA Amplification Kit (Amersham Biosystems, Piscataway, NJ) utilizing Φ 29 DNA polymerase was used to create UUC from genomic DNA. For primary WGA, 1.0 ng of genomic DNA prepared in 1 μ l was amplified in a total volume of 20 μ l following the instruction provided by the kit manufacturer. DNA was diluted with 9 μ l sample buffer containing random primers, heat-denatured at 95 °C for 3 min, cooled to 4 °C, and then mixed with 9 μ l reaction buffer and 1 μ l enzyme mix containing Φ 29 DNA polymerase. All the buffers used were provided as premixed in the kit. The mixture was incubated at 30 °C for 18 h, and then the enzyme was deactivated by heating at 65 °C for 10 min. For nested WGA, 0.1 μ l of the product of primary WGA was amplified in a total volume of 20 μ l with the same protocol as the primary reaction. DNA products synthesized by primary and nested WGA were quantified with UV absorption spectrophotometer after purification by QIAquick PCR purification kit (Qiagen, Valencia, CA). The nested WGA product was electrophoresed on 2% agarose gel, and the DNA length was analyzed.

Creation of UMC by SssI methylase

SssI methylase (New England Biolabs, Beverly, MA), which methylates all cytosine residues within the double-stranded dinucleotide recognition sequence 5'...CG...3', was used to create UMC in accordance with the manufacturer's protocol⁸.

Microsatellite analysis of UUC

To ensure the fidelity and representation of nested WGA, allelic ratios at 34 microsatellite markers (mononucleotide repeat markers BAT25 and BAT26; dinucleotide repeat markers TGFbR2, TP53, D1S228, D2S123, D5S229, D5S346, D6S1678, D6S1700, D6S286, D8S261, D8S262, D8S321, D9S171, D10S197, D10S393, D10S591, D12S1657, D12S1706, D12S327, D12S346, D12S393, D14S51, D14S62, D16S421, D16S422, D17S1832, D17S849, D17S855, D18S61, and D18S70, and tetranucleotide markers D12S1059 and D12S296)¹³⁻¹⁵ of the genomic DNA (template) and UUC (nested WGA product) were compared. Primer sequences were obtained from the National Cancer for Biotechnology Information (NCBI) database. Forward primers were labeled with WellRED dye-labeled phosphoramidites (Beckman Coulter, Fullerton, CA). PCR was performed with 10 ng of genomic DNA or nested Φ 29 WGA product, 2.5 mM Mg²⁺, and 0.2 μ M of each primer in a 10- μ l reaction volume for 36 cycles: 30 s at 94 °C, 30 s at suitable annealing temperature for each primer set, 30 s at 72 °C, and 7-min final extension at 72 °C. The amount and size of the PCR amplicon were determined by capillary array electrophoresis (CAE) with the CEQ 8000XL system (Beckman Coulter). Allelic ratio deviation of nested

WGA products on each heterozygous marker was calculated using the following formula: $\text{Max}(R_G, R_U) / \text{Min}(R_G, R_U) - 1.0$, where R_G , R_U are the CAE intensity ratios of two alleles in genomic DNA and in UUC, respectively.

SBM on UUC and UMC

SBM was applied on UUC and UMC as previously described¹⁶. A 5 µg DNA sample in 46.7 µl was denatured by the addition of 3.3 µl of 3M NaOH and incubated at 37 °C for 15 min. After addition of 520 µl of 2.5M sodium metabisulfite at pH 5.0 and 30 µl of 10 mM hydroquinone, DNA was incubated at 60 °C for 4 h in the dark. The bisulfite-treated DNA was desalted using the Wizard DNA cleanup system (Promega, Madison, WI) and eluted in 50 µl H₂O. DNA was then desulfonated by 5.6 µl of 3M NaOH at 37 °C for 15 min and neutralized by 14 µl of 3M sodium acetate. After ethanol precipitation, the DNA pellet was resuspended in 50 µl of 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0.

Detection of methylation of promoter region of genes

To test the UUC, we demonstrated the methylation status of p16 (INK4a) gene promoter region by SBM direct sequencing and MSP. This gene is one of the most intensely studied tumor suppressor genes in malignant tumors^{5,17}. DNA sequence of p16 gene isoform-1 promoter region with indication of the primers is shown in Fig. 1. We also demonstrated the methylation of promoter regions of seven other genes (RASSF1A (ras association domain family protein 1), hMLH1 (mutL homologue 1), TWIST, ID4 (inhibitor of DNA binding 4), ESR1 (estrogen receptor 1), 14-3-3σ, and MGMT (methylguanine-DNA methyltransferase)) by MSP.

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a c a c g c c t t t g c t g g c a g g c g g g g g a g c g c g g c t g g g a g c a g g g a g g c c g
g a g g g c g g t g t g g g g g g c a g g t g g g g a g g a g c c c a g t c c t c c t t c c t t g c
c a a c g c t g g c t c t g g c g a g g g c t g c t t c c g g c t g g t g c c c c c g g g g g a g a
C C C A A C C T G G G G C G A C T T C A G G G G T G C C A C A T T C G C T A A G T G C T C G G A G T
T A A T A G C A C C T C C T C G A G C A C T C G C T C A C G G C G T C C C C T T G C C T G G A A A
G A T A C C G C G G T C C C T C C A G A G G A T T T G A G G G A C A G G G T C G G A G G G G G C T C
T T C C G C C A G C A C C G G A G G A A G A A A G A G G A G G G G C T G G C T G G T C A C C A G A G
G G T G G G G C G G A C C G C T G C G C T C G G C G G C T G C G G A G A G G G G A G A G C A G G
C A G C G G G C G G G G A G C A G C A T G G A G C C G G C G G C G G G A G C A G C A T G G A
G C C T T C G G C T G A C T G G C T G G C C A C G G C C G G C C C G G G G T C G G G T A G A G G
A G G T G C G G G C G C T G C T G G A G G C G G G G G C G C T G C C C A A C G C A C C G A A T A G T
    
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Fig. 1. DNA sequence of p16 gene isoform 1 promoter region with indication of the primers for methylation detection analyses. Lowercase: upstream of 50' untranslated region; uppercase: exon 1. Open boxes: SBM sequencing primers; solid underlines: methylated-specific MSP primers; and dotted underlines: unmethylated-specific MSP primers. CpG sites are indicated in bold.

For SBM direct sequencing, 1 μ l of SBM UUC was amplified by PCR with the SBM sequencing primers and 2.5 mM of Mg^{2+} in a 50- μ l reaction volume for 36 cycles: 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C, and a 7 min final extension at 72 °C. Purified PCR products were bidirectionally direct-sequenced by CAE using CEQ DYE Terminator Cycle Sequencing kit (Beckman Coulter). The cycling program included 30 cycles: 20 s at 95 °C, 40 s at 55 °C, and 4 min at 60 °C.

For MSP, each primer was designed to cover two or more CpG sites. Unmethylated-specific and methylated-specific PCR was performed on 1 μ l SBM UUC with 0.2 μ M of each primer and 2.5 mM Mg^{2+} in a 10- μ l reaction volume for 36 cycles: 30 s at 94 °C, 30 s at optimized annealing temperature for each primer set, and a 7-min final extension at 72 °C. Forward primers were labeled with WellRED dyelabeled phosphoramidites (Beckman Coulter). PCR products were detected and analyzed by CAE.

Results

Nested WGA product

The amount of UUC created by nested WGA in 20 μ l of reaction volume was 15.9 ± 1.1 μ g (mean $\pm$ SEM, $n = 4$), which included only 5 pg of template genomic DNA (1/200 amount of the initial template DNA). The total amplification ratio was 3.2×10^6 , and the contamination ratio of genomic DNA was 3.1×10^7 . When 100 ng of nested WGA product was used as a template for subsequent analysis, estimated contamination of the genomic DNA was as low as 0.02 copies per reaction, below the minimum detection level of a highly sensitive method such as MSP. When the nested WGA product was electrophoresed on 2% agarose gel, the DNA fragment length was widely distributed but >5 kb fragments were dominant, indicating wide coverage of the genes and their promoter regions (Fig. 2A).

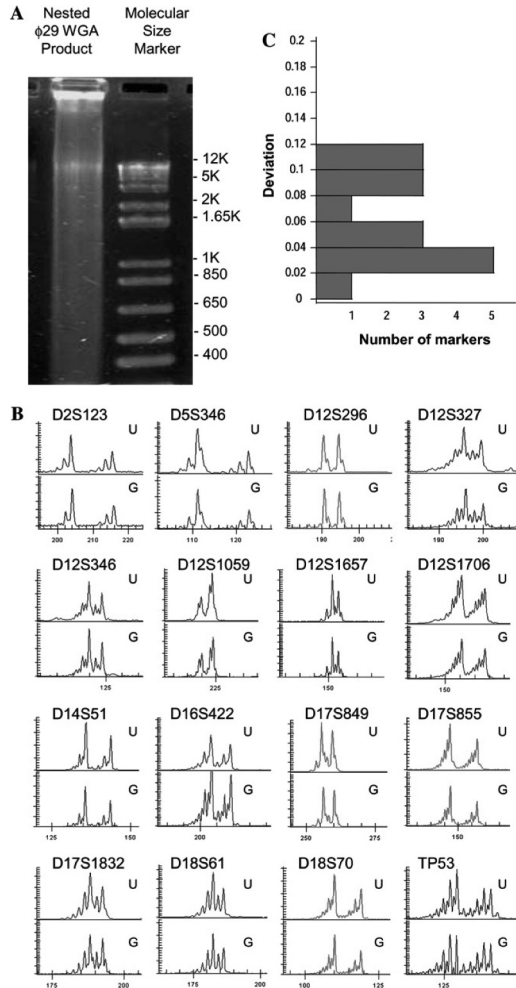
Microsatellite analysis

All 34 microsatellite markers were sufficiently amplified on UUC. When amplicon length and distribution were compared for each marker, all the peaks shown by CAE were equivalent and no aberrant bands were observed. No significant amplification error occurred on the nucleotide repeats, and the amplicon size was completely preserved after nested WGA reactions (Fig. 2B). In 16 heterozygous markers, allelic ratios were highly maintained in all markers; the deviations caused by nested WGA were only $5.9 \pm 0.8\%$ (mean $\pm$ SEM) with a maximum value of 11% in D12S327 (Figs. 2B and C). Thus, the two alleles were equally amplified by nested WGA, and it was demonstrated that Φ 29 WGA achieves highly accurate and homogeneous amplification, even on nested reactions with very low amounts of genomic DNA as a template.

Methylation status of gene promoter regions in UUC

As a demonstration, SBM direct sequencing and MSP at p16 promoter region on the UUC were performed. Sequencing showed that all cytosines of CpG dinucleotides were converted to uracils; thus, there was no methylation on CpG dinucleotides (Fig. 3A). In MSP, only the unmethylated-specific peak was observed (Fig. 3B). Restriction enzymatic digestion

Fig. 2. (A) Nested Φ 29 WGA product electrophoresed on 2% agarose gel along with molecular size marker. DNA fragment lengths were widely distributed, but >5 kb fragments were dominant. (B) CAE results of 16 microsatellite markers showing heterozygosity on genomic DNA among 34 tested markers. Upper figure (U) in each marker is of UUC and lower figure (G) is of genomic DNA used as a template. The vertical axis represents the fluorescent intensity indicating the amount of PCR amplicon and the horizontal axis represents the PCR amplicon size. All the peaks shown by CAE were equivalent and no aberrant bands were observed. Allelic ratios were highly maintained in all markers. (C) Histogram of allelic ratio deviation of UUC; the allelic ratio deviation was $5.9 \pm 0.8\%$ (mean $\pm$ SEM).



study using methylation-specific restriction endonuclease HpaII confirmed that the CpG within the recognition site was not methylated in UUC (data not shown). COBRA analysis using restriction endonuclease HpyCH4 IV further confirmed that specific CpG site in p16 promoter region had no methylation (data not shown). Similarly, MSP analysis of the promoter regions of RASSF1A, hMLH1, TWIST, ID4, ESR, 14-3-3 σ , and MGMT showed only unmethylated-specific peaks (Fig. 3B). In all primer sets, the PCR amplicon length and the shape of the peaks were identical for UUC and PBL DNA. Thus, nested Φ 29 WGA did not affect promoter sequence.

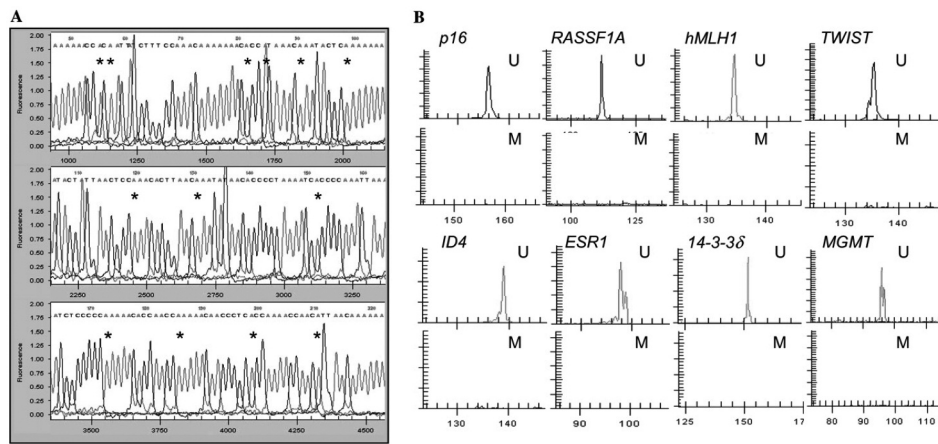


Figure 3. (A) SBM direct sequencing result at p16 promoter region on UUC in reverse direction. It is shown that all the cytosines of CpG dinucleotides in UUC were converted to uracils (shown as A in reverse sequence with *), representing that there was no methylation on CpG dinucleotides. (B) Methylated- and unmethylated-specific MSP for p16, RASSF1A, hMLH1, TWIST, ID4, ESR1, 14-3-3 δ , and MGMT on SBM UUC. Unmethylated-specific peaks were observed in unmethylated-specific MSP in upper figures (U), but no peaks were observed in methylated-specific MSP in lower figures (M). The vertical axis represents the fluorescent intensity indicating the amount of PCR amplicon and the horizontal axis represents the PCR amplicon size.

Discussion

There are two major types of WGA: thermal cycle amplification using thermostable DNA polymerase and continuous amplification at a stable temperature using DNA polymerase such as Φ 29. To make a UUC DNA, the entire genome including GC-rich sequence must be equally amplified. However, thermal cycling methods amplify the genome unequally depending on the distribution of primer annealing sites, and the length of products is relatively short. In addition, because GC-rich sequences are not efficiently amplified⁹, thermal cycling methods may not be adequate for the CpG islands, which are the usual targets of interest for methylation studies. In contrast, a continuous amplification method using Φ 29 DNA polymerase, which synthesizes the DNA strand displacing the bound complementary DNA, has demonstrated that genome representation was comprehensive and estimated to be 99.8% complete, there was no degradation in the accuracy of single nucleotide polymorphism (SNP) genotyping, and the estimated error rate ($9.5 \cdot 10^{-6}$) was equivalent to that for unamplified samples¹². In our results of microsatellite markers, it was demonstrated that Φ 29 WGA achieves highly accurate and homogeneous amplification, even on nested reaction with very low amounts of genomic DNA as a template. Contamination of methylated CpG dinucleotides of the template genomic DNA must be minimized in UUC. However, amplification ratio of Φ 29 WGA is only 10^3 – 10^4 , and it is insufficient because

the subsequent method such as MSP can detect as low as 10^4 level of contaminated methylated DNA⁷. Nested amplification can reduce the percentage of the contaminating genomic DNA used as the template. In this study, a nested Φ 29 WGA amplified the template DNA by a factor of 3.2×10^6 . The final concentration of contaminant was only 0.02 copies for the subsequent reactions, a negligible amount. Nested Φ 29 WGA maintained the allelic ratio and the size of microsatellites, representing the high fidelity of the amplification. As shown in gel electrophoresis, the fragment of DNA synthesized by nested Φ 29 WGA had sufficient length to cover promoter regions, and the CpG islands in the promoter regions were adequately amplified. In addition, results of MSP and SBM direct sequencing demonstrated that the promoter regions were maintained in the nested Φ 29 WGA products. For highly sensitive methods, both negative and positive controls are essential to optimize conditions. UUC is very useful and necessary for methylation studies, especially MSP, which is a sensitive and powerful technique but critical for condition settings. Inappropriate settings easily cause false-positive and/or false-negative results. The designing of MSP primer sets can have difficulties because of restrictions such as limited annealing sites. Optimization of the PCR is essential but can be difficult without adequate controls. In conclusion, the UUC DNA created by nested Φ 29 WGA is practically very useful and highly essential for adequate methylation analyses such as evaluations of promoter methylation status of tumor-related genes in tumor tissues. Contamination ratio of the template genomic DNA in UUC was below the detection limit of MSP or other sensitive methods. Because the UUC contains near complete whole genome sequence and does not contain methylated cytosines, it can be utilized as a standard control material for various genomic methylation analyses.

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CHAPTER 2

Methylation of p16 and Ras Association Domain Family Protein 1a during Colorectal Malignant Transformation

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Abstract

Accurate assessment of gene methylation in formalin-fixed, paraffin-embedded archived tissue (FF-PEAT) by microdissection remains challenging because the tissue volume is small and DNA is damaged. In addition, methods for methylation assessment, such as methylation-specific PCR (MSP), require sodium bisulfite modification (SBM) on purified DNA, which causes major loss of DNA. On-slide SBM, in which DNA is modified *in situ* before isolation of tumor cells, eliminates DNA purification steps and allows histology-oriented assessment of gene methylation. This study describes a protocol and use of on-slide SBM using 20 FF-PEAT of colorectal cancers with intratumoral adenoma components to detect accumulation of gene methylation during colorectal malignant transformation. Deparaffinized tissue sections were incubated in sodium bisulfite solution for 8 hours at 60°C, stained with hematoxylin, and then microdissected. Proteinase K lysate was directly used as a template in subsequent PCR. Using on-slide SBM, 282-bp-long bisulfite direct sequencing was possible. Yield of modified DNA was 2.6-fold greater than standard SBM on average. The mean conversion rate was 97%, and false-positive or false-negative results were not observed in subsequent MSP. Intratumoral heterogeneity by accumulation of p16 and Ras association domain family protein 1a methylation during malignant transformation were shown by MSP comparing cancer with adenoma parts within a single section. On-slide SBM is applicable in most methylation studies using FF-PEAT. It allows detailed, intratumoral analysis of methylation heterogeneity within solid tumors. On-slide SBM will significantly improve our approach and understanding of epigenetic events in minimal disease and the carcinogenic process.

Introduction

In the mammalian genome, 5% of cytosines are modified to 5-methylcytosine¹, and this modification has important regulatory effects on gene expression especially when it involves cytosine-guanine dinucleotide (CpG) rich areas (CpG islands) in promoter regions^{2,3}. Epigenetic gene silencing by promoter hypermethylation of tumor suppressor genes is as significant as deletions or mutations⁴⁻⁶, and it can play a significant role in malignant transformation and immortalization of cells. Therefore, assessment of promoter hypermethylation has become important for understanding the mechanisms of malignant transformation^{7,8}. Sodium bisulfite modification (SBM) of genomic DNA is commonly used to detect methylation of CpG islands in promoter regions. Sodium bisulfite converts cytosines but not 5-methylcytosines to uracils⁹. Therefore, the methylation status reflects the changes in the sequence of the DNA after modification. The modified DNA can be analyzed by sequencing¹⁰, methylation-specific PCR (MSP)¹¹, combined bisulfite restriction analysis¹², high-throughput quantitative methylation assay (MethyLight)¹³, quantitative analysis of methylated alleles¹⁴, and other methods. The most widely available starting material for assessment of solid tumors is formalin-fixed, paraffin-embedded archived tissue (FF-PEAT) used for pathologic diagnosis after surgical removal of the tumor or after tumor biopsy. Use of FF-PEAT for epigenetic analysis, such as MSP, would have significant use to evaluate patients' specimens. As described in a previous report, there are critical issues with SBM of FF-PEAT^{15,16}, formalin fixation causes fragmentation and cross-linking of the DNA that is dependent on the acidity of the formalin and soaking time. In addition, 84% to 96% of the source DNA may be lost during conventional SBM¹⁷. This significant loss of template DNA hampers assessment of methylation; a previous study reported that 50 cycles of thermal cycling in MSP were needed for SBM DNA extracted from a small area of FF-PEAT by microdissection¹⁸. This high number of PCR cycles would detect only a single copy of DNA, which increases the risk of false-positive results due to incomplete SBM, and may not be reproducible. Extracting DNA from a larger tumor area or whole-tumor sections increase yields of DNA would result in more reproducible results, but it increases the risk of contamination with DNA from non-cancer cells. On-slide SBM, in which DNA is modified in the tissues, was initially developed for MSP in situ hybridization¹⁹, but its application has been very limited to date. However, on-slide SBM should minimize loss of DNA because it eliminates DNA purification steps. We have developed and optimized a protocol for on-slide SBM that can be easily adapted in laboratories to do histology-oriented methylation analyses. We validated the use of on-slide SBM using primary colorectal cancers with intratumoral adenoma component. Specificity was assessed by estrogen receptor 1 (ESR1) and thymine-DNA glycosylase (TDG) promoter methylation status. Intratumoral heterogeneity of p16 and Ras association domain family protein 1a (RASSF1a) promoter methylation status was also assessed. The feasibility of direct sequencing of a relatively long PCR product was tested to show that DNA modified by the on-slide SBM technique is degraded to a lesser extent compared with conventional SBM.

To evaluate the yield and conversion rate (i.e., percentage of modified DNA) of on-slide SBM, we have newly developed a highly sensitive quantitative real-time PCR (qPCR) assay for bisulfite-modified and unmodified DNA (i.e., genomic DNA). Alu, which is the most

abundant repeat sequence in the human genome with a copy number of 1.4×10^6 in a cell²⁰, was used as a target of qPCR to maximize sensitivity. It allowed accurate quantification and precise evaluation for samples having very low amount of modified DNA. Bisulfite-modified DNA and the remaining unmodified DNA were separately quantified by Alu-qPCR with specifically designed primer sets.

Materials and Methods

Tissue Specimens

FF-PEAT of colorectal cancers were obtained from 20 patients who underwent colectomy or proctectomy between 1995 and 1998 at Saint John's Health Center (Santa Monica, CA) and consented for research use of their tissue specimens according to the guidelines of Saint John's Health Center/John Wayne Cancer Institute's (Santa Monica, CA) review board. Cases were selected in which the pathologic report described presence of intratumoral adenoma component. All tissue specimens had been fixed in 10% buffered formalin for 24 hours and paraffin embedded. A set of eight colorectal cancer specimens was used to evaluate yield, conversion rate, and a set of three colorectal cancer specimens was used to show feasibility of relatively long sequencing of on-slide SBM DNA. Sections (10 μm) were cut with a microtome from each FF-PEAT block.

On-Slide SBM and Subsequent Microdissection

To evaluate the yield and conversion rate of on-slide SBM, deparaffinized and rehydrated tissue sections were incubated in 0.2mol/L NaOH at room temperature for 15 minutes and then incubated in 3 mol/L sodium bisulfite solution with 0.5 mmol/l hydroquinone (pH 5) in the dark. We tested four incubation settings (60°C for 4 hours, 60°C for 8 hours, 70°C for 4 hours, and 70°C for 8 hours) for three colorectal cancer specimens in the initial evaluation set. Eight colorectal cancer specimens were used with two incubation settings (60°C for 4 hours and 60°C for 8 hours) to compare conversion rate and yield of modified DNA between on-slide SBM and conventional SBM methods. After incubation, sections were rinsed with distilled water, soaked in 0.3 mol/l NaOH for 15 minutes to desulfonate the modified cytosines, and then desalted in distilled water at 60°C for 2hours. After light staining with hematoxylin, 25-mm² cancer tissue was microdissected under a microscope from each section. The microdissected areas of each section were identical for each incubation setting. Microdissected tissues were digested in 50 μl lysis buffer containing 4 μg proteinase K, 2.5% Tween 20, 50 mmol/l Tris, and 1 mmol/l EDTA at 50°C for 5 hours followed by heat deactivation of proteinase K at 95°C for 10 minutes. In each subsequent qPCR, 2 μl lysate was used as a template without DNA purification.

To validate the use of on-slide SBM to study intratumoral heterogeneity in methylation status, paired cancer and adenoma tissues were microdissected from each on-slide SBM section treated at the conditions that were found to be optimal. From each specimen, ~1 mm² tissue was microdissected and consisted of ~2 to 10 ducts of cancer or adenoma tissue. Each specimen was digested in 10 μl lysis buffer; 1 μl lysate was used as a template in subsequent MSP or direct sequencing without DNA purification.

Standard SBM on Purified DNA from Microdissected Tissue

For comparison of standard SBM with on-slide SBM DNA, purified DNA from microdissected FF-PEAT sections was bisulfite modified with standard SBM method as described previously²³. DNA was extracted from the same area (25 mm²) of each tissue section as on-slide SBM. DNA was purified with phenol-chloroform-isoamylalcohol from proteinase K-digested tissues microdissected from deparaffinized sections and modified by sodium bisulfite 60°C for 3 hours with 1 µg salmon sperm DNA as a carrier using the same bisulfite solution as described above. Modified DNA was recovered using the Wizard DNA Clean-Up System (Promega, Madison, WI), desulfonated with 0.3 mol/l NaOH, and resuspended in 50 µL Tris-EDTA buffer; 2 µl of the solution were used as a template in subsequent qPCR.

Agarose-Bead SBM on Microdissected Tissue

Bisulfite modification of DNA in digested tissues trapped in agarose beads²⁴ does not require the DNA purification steps and therefore allows a relatively high recovery rate of DNA from FF-PEAT samples. Same area (25 mm²) of each tissue section was microdissected and treated. For bisulfite modification, digested tissues trapped in 2% agarose beads containing 0.2mol/l NaOH were incubated in bisulfite solution, same as for on-slide and standard SBM, at 50°C for 14 hours. After modification, beads were desalted as described previously (24) and heat diluted up to 50 µl with Tris-EDTA buffer; 2 µl of the diluted agarose gel were directly used as a template in subsequent qPCR without DNA purification.

Quantification of Bisulfite-Modified and Unmodified DNA

To determine the yield and conversion rate of SBM DNA, bisulfite-modified and unmodified DNA was separately quantified by Alu-qPCR. Primers specific for bisulfite-modified Alu sequence independent to the methylation status were 5'-TTGTAATTTAGTATTTGGGAGGT-3' (forward) and 5'-TCACCATATTAACCAACTAATCTC-3' (reverse); their amplicon size was 83 bp. Primers specific for unmodified Alu sequence were 5'-CCTGAGGT CAGGAGTTCGAG-3' (forward) and 5'-CCCGAGTAGCTGGGATTACA-3' (reverse); their amplicon size was 115 bp. The reaction mixture for each Alu-qPCR consisted of SBM DNA template, 0.2 µmol/l forward and reverse primers, 1 unit iTaq DNA polymerase (Bio-Rad Laboratories, Hercules, CA), 0.02 µl fluorescein calibration dye (Bio-Rad Laboratories), and 1x concentration of SYBR Gold (Molecular Probes, Eugene, OR) in a total reaction volume of 20 µl with 5 mmol/l Mg²⁺. Real-time PCR amplification was done with a pre-cycling heat activation of DNA polymerase at 95°C for 10 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 59°C (primer set for modified Alu) or 64°C (primer set for unmodified Alu) for 30 seconds, and extension at 72°C for 30 seconds using iCycler iQ Real-time PCR Detection System (Bio-Rad Laboratories). The absolute copy numbers of modified and unmodified Alu sequence in each sample were determined from the threshold cycle numbers with a standard curve done by serial dilutions of bisulfite-modified or unmodified Alu fragment, which was purified from PCR product and quantified by UV-absorption. Triplicate reactions of qPCR were done, and each reaction plate included a negative control (no template). PCR products were electrophoresed on 2% agarose gels to confirm product size and specificity of the PCR. The relative yield was calculated as $Q_{os} /$

Q_{std} for on-slide SBM and $Q_{\text{ag}} / Q_{\text{std}}$ for agarose-bead SBM, where Q_{std} , Q_{os} , and Q_{ag} are the absolute copy numbers of modified Alu of standard, on-slide, and agarose-bead SBM, respectively. The conversion ratio was calculated as $Q_{\text{mod}} / (Q_{\text{mod}} + Q_{\text{unmod}})$, where Q_{mod} and Q_{unmod} are the absolute copy numbers of modified and unmodified Alu, respectively. Cross-reaction of Alu primers on the salmon sperm DNA used as a carrier in the standard SBM was assessed using a blank sample prepared without tissue DNA.

Bisulfite Direct Sequencing for p16 Gene Promoter Region

To show the feasibility of on-slide SBM for bisulfite direct sequencing of relatively long target, we sequenced 282 bp of the modified p16 gene promoter, which is usually difficult for DNA from FF-PEAT modified by standard SBM. Bisulfite direct sequencing was done as described previously²⁵. The primers were 5'-GGGTAGGTGGGGAGGAGTTTAGTTT-3' (forward) and 5'-AATAACCAACCAACCCCTCCTCTTT-3' (reverse). PCR amplification was done in a 50- μ l reaction volume using 1- μ l template with a precycling heat activation of DNA polymerase at 95°C for 10 minutes followed by 36 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds followed by final extension at 72°C for 7 minutes with 2.5 mmol/l concentration of Mg^{2+} and 0.2 μ mol/l of each primer concentration. After PCR products were run on agarose gels and purified, they were directly sequenced using CEQ Dye Terminator Cycle Sequencing kit (Beckman Coulter, Inc., Fullerton, CA) according to the manufacturer's instructions. Cycling program includes 30 cycles of denaturation at 95°C for 20 seconds, annealing at 55°C for 40 seconds, and extension at 60°C for 4 minutes. Sequences were read by CEQ 8000XL CAE System (Beckman Coulter).

MSP for ESR1, TDG, p16, and RASSF1a

Methylation status of CpG islands in ESR1, TDG, p16, and RASSF1a gene promoter regions of paired cancer and adenoma tissues was assessed by MSP. Forward primers of MSP were labeled with D2, D3, or D4 WellRED dye-labeled phosphoramidites (Beckman Coulter). The methylation-specific primers for ESR1 were 5'-D4-TAAATAGAGATATATCG-GAGTTTGG TACG-3' (forward) and 5'-AACTTAAAATAAACGCGAAAAACGA-3' (reverse); the unmethylation-specific primers for ESR1 were 5'-D3-TAAATAGAGATATATTGGAGTTT GGTATGG-3' (forward) and 5'-AACTTAAAATAAACACAAAAAACAAA-3' (reverse). The methylation-specific primers for TDG were 5'-D4-TGAGCGTGATTGGGTTGTTTTAT ATTAC-3' (forward) and 5'-TTCTACCGTCCCCACGA-3' (reverse); the unmethylation-specific primers for TDG were 5'-D3-AGTGTGATTGGGTTGTTTTATATTATGT-3' (forward) and 5'-CTTCTACCATCCCCACAAA-3' (reverse). The methylation-specific primers for p16 were 5'-D4-TTATTAGAGGGTGGGGCGGATCGC-3' (forward) and 5'-CG ATTTGGGTCGCGGTCTGTGG-3' (reverse); the unmethylation-specific primers for p16 were 5'-D2-TATTAGAGGGTGGGGTGGATTGT-3' (forward) and 5'-TGATTTTGGGTTG TGGTTGTGG-3' (reverse). PCR conditions were optimized to achieve highest sensitivity without false-positive results using universal methylated control DNA and universal unmethylated control DNA as described previously²⁵. The optimized annealing temperatures for methylated-specific primers for ESR1, unmethylated-specific primers for ESR1, methylated-specific primers for TDG, and unmethylated-specific primers for TDG, methylated specific

primers for p16, and unmethylated-specific primers for p16 were 61°C, 58°C, 62°C, 61°C, 62°C, and 61°C, respectively. Primers and PCR settings used for amplification of the RASSF1a promoter region were published previously²³. PCR amplification was done in a 10- μ l reaction volume using 1- μ l template with a precycling heat activation of DNA polymerase at 95°C for 10 minutes followed by 36 cycles of denaturation at 95°C for 30 seconds, annealing at optimized annealing temperature for 30 seconds, and extension at 72°C for 30 seconds followed by final extension at 72°C for 7 minutes with 2.5 mmol/l concentration of Mg²⁺ and 0.2 μ mol/l of each primer concentration. PCR products were detected and analyzed by CEQ 8000XL CAE system as described previously²⁶.

Results

Effect of On-Slide SBM on Microscopic Histology of Tissue Sections

Because on-slide SBM uses alkaline pretreatment for denaturation of DNA, potent acid treatment for modification of cytosines at relatively high temperatures for long durations, and alkaline treatment for desulfonation of cytosines, we expected some deterioration of microscopic tissue architectures. However, as shown in Fig. 1, tissue architecture was maintained after on-slide SBM at optimal conditions of 60°C for 8 hours, and there was no impairment of pathologic assessment after hematoxylin staining. Consequently, microdissection could be precisely done without difficulty. H&E and methyl green staining was also done on post on-slide SBM slides and showed similar quality (data not shown).

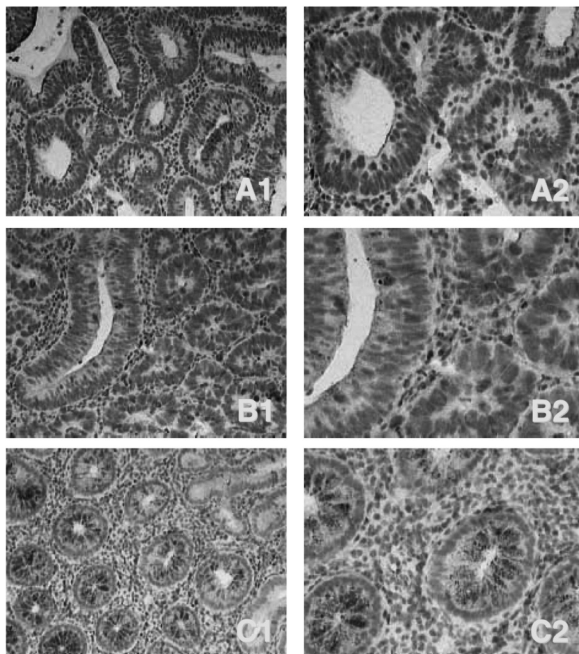


Figure 1. Microscopic photographs of three tissue sections after on-slide SBM treatment and hematoxylin staining. Magnification, 100 (A1, B1, and C1) and 200 (A2, B2, and C2).

Bisulfite Direct Sequencing of p16 Gene Promoter Region

Bisulfite direct sequencing of a 282-bp amplicon in the p16 gene promoter region was done in an initial evaluation set of three colorectal cancer specimens comparing two incubation temperatures of on-slide SBM. Sequencing of on-slide SBM samples incubated at 70°C for 4 or 8 hours was unsuccessful. This suggests that template DNA may be damaged and truncated during incubation at 70°C, thus not suitable for assessment. In contrast, on-slide SBM samples incubated at 60°C for 4 or 8 hours were successfully sequenced (Fig. 2). All cytosines in the sequencing results were correctly converted to uracil with incubation at 60°C for 8 hours. Therefore, incubation at 60°C was used for on-slide SBM in the subsequent assessments. Direct sequencing of DNA after standard SBM or agarosebead SBM was unsuccessful with the same sequencing protocol.

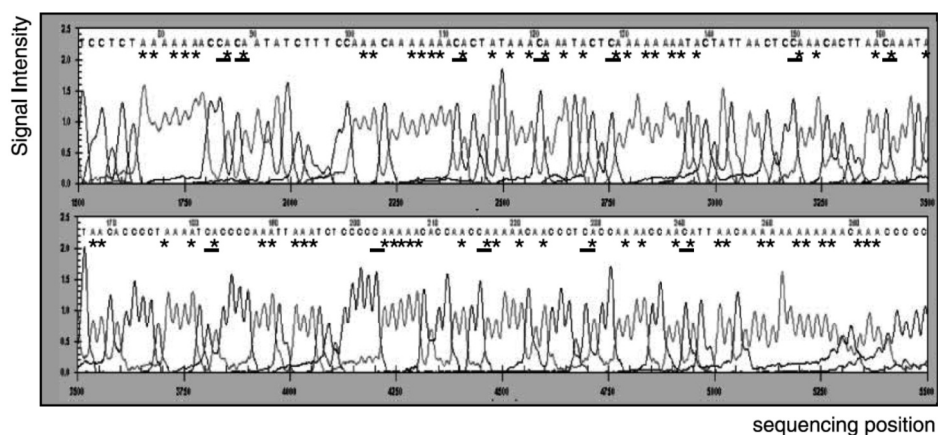


Figure 2. Representative standard sequencing diagram for bisulfite direct sequencing of p16 gene promoter region after on-slide SBM DNA with incubation at 60°C for 8 hours depicting the high quality of modified DNA. Bisulfite direct sequencing of a 282-bp amplicon was made possible by on-slide SBM from 1-mm² microdissected tissue of 10-Am FF-PEAT section of a colorectal cancer. All cytosines in this sequence (*) were converted to uracil, shown as “A” in reverse strand. Signals of false conversion were not observed. CpGs are underlined. The p16 gene promoter region of this colorectal cancer is not methylated.

The Yield and Conversion Rate of Modification

The yield and conversion rate of modification was evaluated using eight tissue samples for on-slide, standard, and agarosebead SBM by assessing the copy numbers of modified and unmodified Alu repeat sequences. Each sample was assessed in triplicate, and the median intersample coefficient of variance for modified and unmodified DNA was 6.8% and 11.2%, respectively. The yield of modified DNA increased with the duration of incubation of on-slide SBM. When modified Alu copy numbers of on-slide and standard SBM were compared, the relative yield of modified DNA by on-slide SBM was higher by a mean factor of 1.3

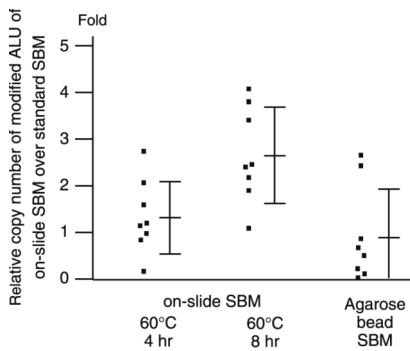


Figure 3. Relative yield of modified DNA of on-slide and agarosebead SBM over standard SBM, calculated as the ratio of absolute copy numbers of modified Alu. On-slide SBM at incubation settings of 60°C for 8 hours had the highest relative yield, which was 2.6 ± 1.0 -fold (mean $\pm$ SD) greater than the yield of standard SBM.

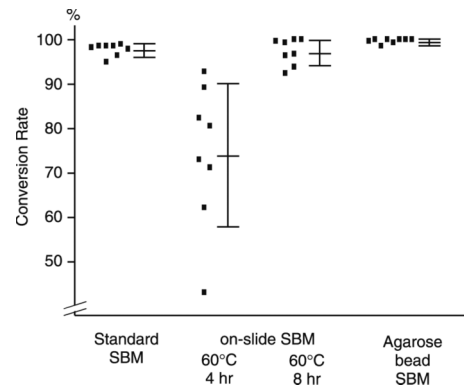


Figure 4. Conversion rates of standard, on-slide, and agarose-bead SBM, calculated as the ratio of absolute copy numbers of bisulfite-modified Alu over total absolute copy numbers of modified and unmodified Alu. On-slide SBM at incubation settings of 60°C for 8 hours had similar conversion rate as standard SBM.

± 0.8 and 2.6 ± 1.0 (mean $\pm$ SD) at incubation settings of 60°C for 4 hours and 60°C for 8 hours, respectively (Fig. 3). The relative yield of modified DNA by agarose-bead SBM was only 0.9 ± 1.0 (mean $\pm$ SD) after 14 hours of incubation at 50°C. The conversion rates of modified DNA by on-slide SBM were $75.4 \pm 15.2\%$ and $97.1 \pm 2.7\%$ (mean $\pm$ SD) at incubation settings of 60°C for 4 hours and 60°C for 8 hours, respectively (Fig. 4). Conversion rates increased with increasing duration of incubation. The conversion rate of on-slide SBM with 4 hours of incubation was obviously low for specific assessment of methylation status, but it was satisfactory and similar to the standard SBM method with 8 hours of incubation. The conversion rate of standard SBM was $97.6 \pm 1.3\%$ after 3 hours of incubation at 60°C. Assessment of cross-reaction of Alu primers on the salmon sperm DNA used as a carrier in the standard SBM revealed that it contributed only $<0.1\%$ of the total copy numbers. The agarose-bead SBM method had a conversion rate of $99.4 \pm 0.5\%$ after 14 hours of incubation at 50°C.

MSP for ESR1, TDG, p16, and RASSF1a

To evaluate the possibility of false-positive or false-negative MSP results after on-slide SBM, we assessed the methylation status of CpG islands in ESR1 and TDG gene promoter of 16 pairs of cancer and adenoma tissues. All showed methylation of ESR1 (Fig. 5A), and none showed methylation of TDG (Fig. 5B). These results indicate that on-slide SBM did not

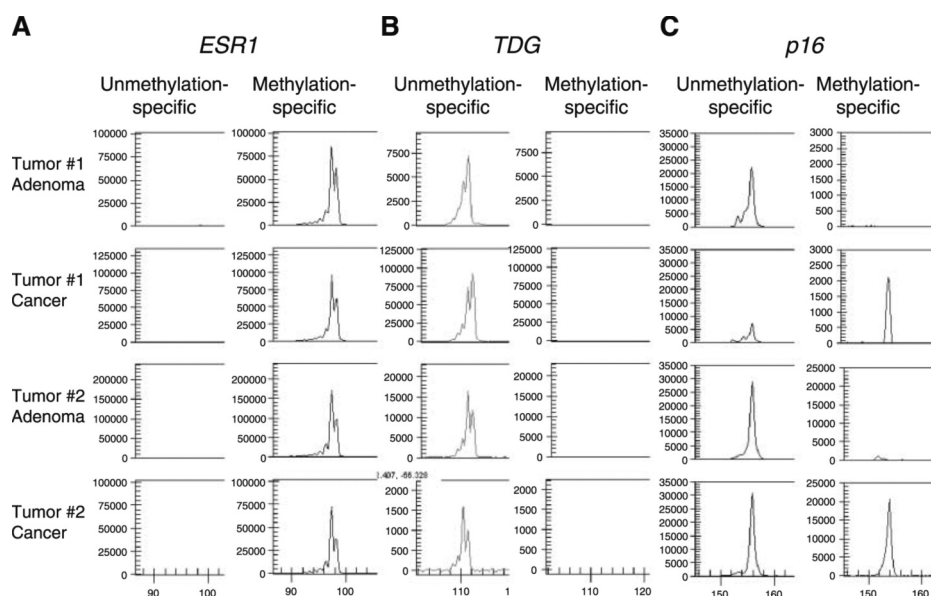


Figure 5. Representative MSP results for promoter regions of *ESR1* (A), *TDG* (B), and *p16* (C) genes after on-slide SBM of 16 pairs of cancer and adenoma tissues. For each MSP, 1-mm² tissue from 10- μ m FF-PEAT section was microdissected and 1 of 10 tissue lysate was used as a template. X axis, amplicon size; Y axis, relative amounts of the PCR amplicons.

cause false-positive or false-negative results by MSP. Methylation status of the CpG island in the *p16* and *RASSF1a* gene promoter region was assessed to show the use of on-slide SBM for assessment of intratumoral heterogeneity of epigenetic alterations. MSP results from cancer and adenoma parts within the same tissue section were compared. Among the 16 evaluated pairs, 4 showed methylation of the *p16* promoter in both the cancer and the adenoma parts, and 3 showed methylation only in cancer part (Fig. 5C). These results show that certain colorectal cancers arisen from adenoma acquire *p16* promoter hypermethylation during malignant transformation, and others acquire it at an earlier stage. Methylation of *RASSF1a* promoter was assessed for a total of 20 pairs of colorectal cancers and adenomas. One sample showed methylation of *RASSF1a* promoter in both the cancer and the adenoma parts, and two showed methylation only in cancer part among the 19 informative pairs. Specific involvement of *RASSF1a* in malignant transformation of certain colorectal cancers was shown. On-slide SBM was proved to be a powerful tool for precise assessments of gene methylation status, such as intratumoral heterogeneity.

Optimization on Glass Slides

Because tissue sections did not adhere consistently enough to noncoated glass slides during on-slide SBM treatment, we initially tested the adhesive-coated, positive-charged slides (Superfrost Plus microscope slides, Fisher Scientific, Pittsburgh, PA) that are commonly used for immunohistochemistry. However, long incubation periods at increased temperatu-

res in acidic or alkaline solutions sometimes caused tumor tissue sections to detach, and experiments had to be repeated. Therefore, we tested silane coated slides (Tekdon, Inc., Myakka City, FL) to improve the robustness of the protocol on 20 colorectal cancer tissue samples. Consequently, none of the tissue sections detached during the on-slide SBM process. The yield of modified DNA and conversion rate was equivalent to the other kinds of glass slides (data not shown). Because of the improvement of robustness of the protocol, we advocate use of silane coated slides for on-slide SBM.

Discussion

As shown by our results, we were able to study malignant tumor progression by assessing intratumoral heterogeneity in silencing by hypermethylation using a single section of FF-PEAT. On-slide SBM technique is widely applicable because FF-PEAT is the most abundant tissue source for cancer-related studies or any other disease-related studies. The use of on-slide SBM could greatly enhance our knowledge of the relation between histopathologic observations and epigenetic events. To date, to study such phenomena *in vivo* has been difficult because of technical limitations of conventional SBM methods. By simply applying the SBM step *in situ* previous to DNA isolation from the cells of interest, we were able to obtain sufficient amounts of DNA to do PCR analyses. Consequently, we could provide evidence that epigenetic changes of p16 and RASSF1a might be involved in a multistep manner in the progression of the adenomas to colorectal cancers similar to the structural DNA changes²¹.

The success of methylation analysis depends on yield of modified DNA and completeness of conversion. When the yield of modified DNA is low, it becomes difficult to detect the methylation status of small amounts of tissue. If the conversion rate is low, false-positive results for methylation can be observed in unmethylated DNA by PCR-based nonquantitative methods because of misgenerated DNA coincidentally having the similar sequence of methylated DNA. Our results showed that TDG, which was unmethylated by standard SBM in a pilot study using 26 colorectal cancer tissues and six colorectal cancer cell lines, was unmethylated by on-slide SBM in all 16 pairs of cancer and adenoma tissues. In addition, ESR1, which is reportedly methylated in all colorectal cancers²², was methylated by on-slide SBM in all 16 pairs. Therefore, on-slide SBM is accurate enough for highly-sensitive methods, such as MSP, and does not cause false-negative or false-positive results. After incubation at 60°C for 8 hours, the conversion rate of on-slide SBM was similar to that of standard SBM. Furthermore, the yield was greater than that of agarose-bead SBM, which is commonly used for specimens containing minimal DNA, such as microdissected FF-PEAT tissue or blood serum.

We did not pretreat the tissue samples with a digestive enzyme, such as pepsin or proteinase K, that elevates permeability of reaction solution routinely done in a previous MSP *in situ* hybridization report¹⁹, because predigestion can damage the microscopic tissue structure. Moreover, in our preliminary assessment, predigestion did not significantly increase the conversion rate or the yield of modified DNA (data not shown). Eliminating the predigestion step maintains the microscopic morphology of the tissue during SBM and

allows subsequent hematoxylin staining and microdissection.

We initially planned to use a SBM direct sequencing technique to evaluate the conversion rates between three SBM methods. Assessment of 282 bp of the p16 promoter region of samples treated with the on-slide SBM protocol showed a complete, 100% conversion. Therefore, doing sequencing on modified DNA of a limited number of samples to evaluate the conversion rate was considered not fit to assess subtle differences among the three SBM methods. In addition, our preliminary assessment of SBM DNA revealed that unmodified cytosines are not randomly scattered but exist as clusters in the genome (data not shown) probably because of regional protection from bisulfite, such as fixed double helix. Therefore, we aimed to develop an assay to quantify bisulfite-modified and unmodified DNA using Alu-qPCR for evaluation of yield and conversion rate of SBM DNA. This assay can be widely used as a tool for preevaluation of quality of SBM DNA. In addition, it can be widely used to standardize the amount of template SBM DNA in each reaction of methylation assessment. Using this technique, we showed that on-slide SBM yielded 2.6 times more modified DNA than standard SBM. This is likely caused by loss of DNA in purification steps before and during the standard SBM. The high yield of on-slide SBM is a great advantage when the target tissue is small. In addition, on-slide SBM allowed 282-bp bisulfite direct sequencing from a very small amount of FF-PEAT, reflecting the high quality of modified DNA.

Total reagent cost and labor cost for on-slide SBM were relatively low because it does not require DNA purification. This is particularly advantageous when multiple areas of a tissue section are assessed simultaneously because DNA modification is completed before microdissection. Another advantage of on-slide SBM is consistency of modification conditions, such as incubation temperature, incubation duration, and concentration of bisulfite within a slide. Therefore, on-slide SBM should be especially suitable for assessments of intratumoral heterogeneity.

In conclusion, tissue architecture was maintained during onslide SBM treatment with 8-hours of incubation at 60°C in 3 mol/L sodium bisulfite solution without predigestion. Yield of modified DNA was 2.6-fold greater than that of standard SBM. Bisulfite direct sequencing of a 282-bp amplicon was possible. Conversion rate was 97.1%, and no false-positive or false-negative results in the subsequent MSP for ESR1 and TDG were observed. Intratumoral heterogeneity of p16 and RASSF1a gene methylation representing pathologic malignant transformation was shown. On-slide SBM should be applicable in most methylation studies using FF-PEAT especially in assessments of intratumoral heterogeneity in solid tumors. The approach provides a unique opportunity to assess retrospective archived procured tissues from clinical trials in which there is limited tissue sections availability.

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CHAPTER 3

Assessment of Methylation Events During Colorectal Tumor Progression by Absolute Quantitative Analysis of Methylated Alleles (AQAMA)

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Abstract

To date, the epigenetic events involved in the progression of colorectal cancer (CRC) are not well-described. To study, in detail, methylation during CRC development in high risk adenomas, we developed an assay combining *in situ* (on-slide) sodium bisulfite modification (SBM) of paraffin-embedded archival tissue (PEAT) sections with absolute quantitative assessment of methylated alleles (AQAMA). We tested the performance of the assay to detect methylation level differences between paired pre-malignant and malignant CRC stages. AQAMA assays were used to measure methylation levels at MINT (methylated in tumor) loci 1, 2, 12, and 31. Assay performance was verified on cell line DNA and standard cDNA. On-slide SBM, allowing DNA methylation assessment of 1-2 mm² of PEAT was employed. Methylation levels of adenomatous and cancerous components within a single tissue section in 72 CRC cancer patients were analyzed. AQAMA was verified to accurately assess CpG island methylation status in cell lines. The correlation between expected and measured cDNA methylation levels was high for all four MINT AQAMA assays ($R \geq 0.966$, $P < 0.0001$). Total methylation levels at the 4 loci increased in 11% and decreased in 36% of specimens comparing paired adenoma and cancer tissues ($P < 0.0001$ by Kolmogorov-Smirnov test). Single-PCR AQAMA provided accurate methylation level measurement. Variable MINT locus methylation level changes occur during malignant progression of colorectal adenoma. Combining AQAMA with on-slide SBM provides a sensitive assay that allows detailed histology-oriented analysis of DNA methylation levels, and may give new, accurate insights into understanding development of epigenetic aberrancies in CRC progression.

Introduction

Cytosine-5 of CpG dinucleotides is the unique target of methyl-group placement in mammals¹. CpG methylation is a heritable mechanism to assist in silencing of noncoding DNA in mammals². In cancer, dense methylation of a gene's promoter region, or the region in the vicinity of the 5' region of a gene's open reading frame, can silence expression of genes involved in cancer-related processes³⁻⁵. Tumor-specific DNA methylation events have been demonstrated in a great variety of human cancers and can encompass both loss and gains in methylation⁶. Currently, the clinical utility of detecting CpG methylation status in primary tumors for the management of cancer patient treatment is being evaluated as a useful surrogate marker for disease parameters. Some studies have successfully shown clinical correlates and/or prognostic value⁷⁻⁹. Epigenetic changes may be important as signatures of tumor progression or prognosis, and they may become potential therapeutic targets. Studying epigenetic changes during malignant tumor development would provide valuable additive information on tumor specificity and genesis of key methylation aberrancies.

Recently, studies in colorectal cancer (CRC) have shed new light on the macro- and microscopic pathways involved in the transition from normal epithelium to adenomatous polyps to invasive cancer^{10, 11}. Novel subgroups of colorectal adenomas were identified, indicating differential pathways of CRC development. On the molecular level, Vogelstein et al.¹² reported specific genomic mutations associated with CRC carcinogenesis. On the epigenetic level, it is known that aberrant DNA methylation is present at the earliest dysplastic stages, as well as in malignant tumors¹³. How levels of methylation develop during CRC formation remains uncertain. In general, the molecular events involved in CRC development and progression are still not clearly validated. To investigate this, CRC specimens harboring adenomatous cell components belonging to the precursor lesion would provide an attractive study model. This direct comparison of the pre-malignant lesion with the associated cancer would enable paired analysis of specific events during malignant progression. The adenomatous cells analyzed would represent relevant, high risk cancer precursors, while most studies use randomly selected colorectal adenomas with an unknown likelihood to develop into cancer. We have previously described an approach that enables this direct comparison in CRC paraffin-embedded archival tissue (PEAT) sections by employing *in situ* sodium bisulfite modification (on-slide SBM) of the DNA¹⁴. Adding a quantitative method to evaluate PEAT sections would allow accurate analysis of epigenetic events related to tumor histopathologic changes. To date, such detailed studies have been challenging, as reported studies often fail to microscopically confirm the selection of tumor cells for nucleic acid isolation. On-slide SBM enables DNA methylation assessment of tissue areas of 1-2 mm² in size with DNA yields 2.5 to 4 times higher, and similar efficiency of SBM, compared to standard SBM protocols. Assessment of small areas of tissue allows for more homogenous tumor sample DNA by reducing the risk of selecting uninvolved tissue areas, such as bowel musculature or serosa, especially compared to DNA isolated from whole tissue sections. Using on-slide SBM with absolute quantitative PCR methods would, therefore, give a more reliable representation of methylation levels in specifically defined small areas of tissue.

Advances in sequence detection technology have been made with the addition of minor groove binder (MGB) molecules to Taqman[®] probes. MGB probes have been tested

to be more sequence-specific than standard DNA probes, especially for single base pair mismatches at elevated PCR extension temperatures^{15, 16}. Zeschnigk et al.¹⁷ applied these improved probe qualities to design a fully quantitative, real-time PCR assay for methylation level measurement, QAMA (quantitative assessment of methylated alleles, **figure 1**).

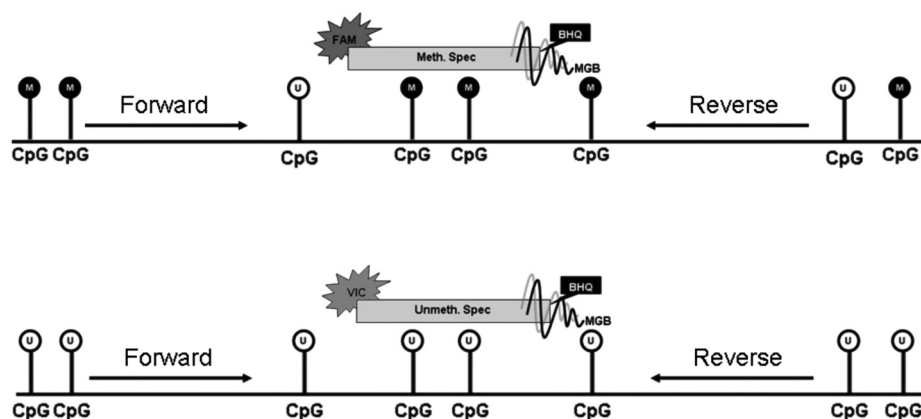


Figure 1. Schematic representation of the AQAMA assay. A universal primer set amplifies a target sequence. **A.** A methylation-specific probe with FAM-labeled reporter; BHQ, and MGB molecule recognizes sample DNA showing hypermethylation. **B.** An unmethylated-specific probe with VIC-labeled reporter; BHQ, and MGB molecule recognizes unmethylated sample DNA.

This method was designed as a relative quantification containing a mathematical derivation using the methylated and unmethylated fluorescent signal threshold value as input. In this study, we used an absolute quantitative version of QAMA (AQAMA) with cDNA standard curves to provide better internal assay control. As methylation biomarkers, we selected four MINT (methylated in tumor) loci, CpG-rich regions^{1, 2, 12 and 31}, as they have been previously demonstrated to become methylated in a tumor-specific and, recently, in an adenoma-specific manner in CRC¹⁸. We demonstrated the accuracy of methylation level assessment of AQAMA alone in evaluating the combination of AQAMA and on-slide SBM to detect changes in methylation levels between paired pre-malignant and malignant CRC cells.

Materials and Methods

Cancer cell lines and patient specimens

For assay validation, DNA was isolated from 8 cancer cell lines obtained from the American Type Culture Collection (ATCC, Manassas, VA); gastric cancer (AGS, SNU-1 and KATO-III), CRC (SW480, SW620, DLD-1, HT-29, Colo320DM and LoVo). All cell lines were cultured and maintained according to the ATCC recommendations. Additionally, we obtained DNA from 2 gastric cancer cell lines (RL-0380 and FN-0028) from the John Wayne Cancer

Institute (JWCI) cell line bank. Seventy-two CRC PEAT blocks were obtained from the surgical pathology department of Saint John's Health Center (SJHC). All human specimens were collected under research protocols reviewed and approved by the combined institutional review board of SJHC/JWCI.

DNA preparation, quantitation and SBM

Genomic DNA from cell lines was isolated as previously described³² with DNAzol (Molecular Research Center, Cincinnati, OH), and quantified and assessed for purity with UV-spectrophotometry. DNA from PEAT was modified according to our previously published protocol¹⁴. Briefly, from each tissue block, a single 4µm section was cut and stained by hematoxylin and eosin (HE). Seven µm PEAT sections were cut consecutively and mounted on adhesive silane-coated slides for DNA studies. Adenomatous and cancer tissue components were identified and marked on the HE stained section by an expert surgical pathologist (R.R.T.). Sections for DNA studies were deparaffinized, soaked in 0.2 M NaOH for 15 min at room temperature, incubated for 8 hr in sodium bisulfite solution at 60°C, rinsed twice with H₂O, soaked in 0.3 M NaOH for 10 min, and desalted in ddH₂O for 2 hr at 60°C. Subsequently, sections were lightly stained with hematoxylin and specific tissue areas were carefully isolated by manual dissection under an inverted light microscope. The isolated tissue was digested in 30 µl lysis buffer containing proteinase K and tween-20 at 50°C for 16 hr. The proteinase K enzyme was then denatured at 95°C for 15 min and the lysate was stored at -30°C. For cell line DNA, SBM was performed on 1 µg of DNA as described previously (33).

AQAMA assay design

Four sets of PCR primers and probes were designed for SBM converted sequences. For a single marker, the assay contains four oligonucleotides. One forward (5') and one reverse (3') primer will amplify the target sequence independent from the markers methylation status, as they do not anneal to any CpG's. The methylation status is assessed by two minor groove binder (MGB)-molecule containing probes (Applied Biosystems, Foster City, CA), one methylation-specific and one unmethylated-specific. Forward and reverse primer sets were designed using Primer 3 software (online at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The MGB probes were designed with Primer Express software (version 2.0, Applied Biosystems) with the MGB probe test document according to the recommendations. Probe length was as short as possible (≥13bp's) while keeping the annealing temperature and GC-percentage of both the methylated and unmethylated probe as similar as possible. Methylated probes were FAM(6-carboxyfluorescein)-labeled, and unmethylated probes were VIC[™]-labelled for optimal discrimination of the two fluorescent signals by the detection system. Black hole quenchers (BHQ) were used to silence the probe fluorescent signal when not hybridized. Selected markers were "methylated in tumor" loci MINT1, 2, 12 and 31. The 5' primer, 3' primer, methylation-specific probe and unmethylated-specific probe are listed as follows, respectively: MINT1 (GGTTGGGTATTTGGATT-TATATTTT, TTCTTTCAAACCTCTCTCAACACTTACT, FAM-5'-AAATCCCCGCCGAAA-3'-MGB-BHQ, VIC-5'-AAAATCCCCACCAAAA-3'-MGB-BHQ), MINT2 (GTGGAAAGTGTTAG-AAAAATGTGTTGTA, TCAACACTTTAACAAAATCCAAAATC, FAM-5'TTTCGTGCAAT-

TTT-MGB-BHQ, VIC-5'-TTTTTTTGTGGAATTTTAG-MGB-BHQ), MINT12(GGGTTTTAG-TTTTGAGGATTAGG, CAAAACCATATCTAAATCACTAACCTT, FAM-5'-AACGACCG-CAAACA-MGB-BHQ, VIC-5'-CCAACAACCACAAAAC-3'-MGB-BHQ), MINT31 (TAA-AGTGAGGGGTGGTGATG, AAAAACACTTCCCCAACATCT, FAM-5'-AGGTTTCGTCTG-TGTTT-3'-MGB-BHQ, VIC-5'-AGGTTTGTGTGTTTAT-3'-MGB-BHQ).

AQAMA PCR

One μ l of modified DNA from cell lines or 1 μ l of digested tumor tissue DNA was amplified in a total volume of 10 μ l on a 384-well plate using fluorescence-based, real-time PCR with the ABI prism 7900HT Sequence Detection System (Applied Biosystems) and SDS software version 2.2.2. The reaction mixture for each AQAMA PCR consisted of DNA template, 0.4 μ M each of forward primer and reverse primer, 1.4 U of *iTaq* DNA polymerase (Bio-Rad Laboratories, Hercules, CA), 350 μ M of each dNTP, and 0.025 pmol of each MGB-probe with 5 mM Mg^{2+} . The master mix contained ROX(6-carboxy-X'-rhodamine)-dye for passive reference fluorescence. Samples were amplified with a pre-cycling hold at 95°C for 10 min to heat-activate DNA polymerase, followed by 40 cycles of denaturation at 95°C for 15 sec, and annealing and extension at 60°C for 1 min for all MINT loci. The final value of data analysis is expressed as a sample's methylation index [MI = methylated copy number / (methylated copy number + unmethylated copy number)]. Sample DNA was added to each reaction plate as controls for specificity of the methylation-specific (AGS and Raji DNA) and unmethylated-specific probe (RL-0380, FN-0028 DNA and donor PBL DNA). PCR and bisulfite reagent controls for non-specific amplification are also included in each plate. Equal PCR efficiency of the methylated and unmethylated reactions was controlled by a duplicated sample that contained equal amounts of methylated and unmethylated cDNA standard.

AQAMA DNA standard construction

The standard curve for quantifying methylated and unmethylated copy numbers was established by amplifying five-aliquot duplicates of templates with known copy numbers (10^5 to 10^1 copies). To obtain high-quality, homogeneous, and consistent DNA standards, we synthesized DNA constructs as follows. We selected cell lines that were confirmed by MSP or bisulfite sequencing to be methylated or unmethylated at the target MINT locus. Regular PCR with only the AQAMA forward and reverse primer on the selected cell line DNA as a template was performed in a 50 μ l reaction volume for 35 cycles, and the product was run on a 2% agarose gel. Specific amplification was confirmed by visualization of a single band. The band was cut out and DNA was extracted using the QIAquick gel extraction method (Qiagen, Valencia, CA) according to the manufacturer's instructions. The completely methylated and unmethylated PCR product was ligated into a pCR 2.1-TOPO cloning vector (Invitrogen, San Diego, CA), the clones were transformed into *Escherichia coli* DH5-cells, and cultures were expanded as described previously³⁴. Plasmids containing the target gene were purified and quantified by UV-spectrophotometry.

MINT locus CpG methylation status confirmation

To assess, non-quantitatively, the CpG methylation status of the MINT loci we employed CAE-MSP as described^{7, 35}. The MINT locus methylation-specific forward (MF), methylation-specific reverse (MR), unmethylated -specific forward (UF) and unmethylated-specific reverse (UR), primers are listed here, respectively;

MINT1, 5'-TTGTTAGCGTTTGTATTTTTTACGT-3' (MF), 5'-AATTACCTCGATAACTTATT-TA CTACGAT-3' (MR), 5'-AGGTTTTTTGTAGTGTGTATTTTTTAT-3' (UF) and 5'-AA AATTACCTCAATAACTTATTTACTACAA-3' (UR);

MINT2, 5'-CGTCGAATTTTAGTA TTAAAGTTCGT-3' (MF), 5'-AATAATAACGAC-GATTCCGTACG-3' (MR), 5'-TTTTGTTGAATTTAGTATTTAAGTTTGT-3' (UF) and 5'-AATAATAACAACAATTCCATACACC-3' (UR);

MINT12, 5'-GTTTTTTCGTAGATTGTGTTTGC- 3' (MF), 5'-CGTTTTATTTAATTTAA-AATCCGAA-3' (MR), 5'-GGTTTTTTTGTAGATTGTGTTTGTG-3' (UF) and 5'-AAAAC-ATTTTATTTAATTTAAATCCAA A-3';

MINT31, 5'-ATATAATTTGTGTATGGATTCGGC-3' (MF), 5'-AATTAAATCGTCT-CAATTCCCG-3' (MR), 5'-ATAA TTTTGTGTATGGATTGGTGA-3' (UF) and 5'-TTAAAT-CATCTCAATTCCCACC-3' (UR). Primers were dye-labeled with different labels for methylation- and unmethylated-specific sets so that PCR products of the predicted base-pair size could be detected by the CEQ 8000XL capillary array electrophoresis (CAE) system (Beckman Coulter, Inc., Fullerton, CA) with CEQ 8000 software version 6.0 (Beckman Coulter). A methylation index was calculated from the detected PCR product signal intensities at the predicted base-pairs size as $[MI = \text{signal intensity methylated PCR product} / (\text{signal intensity methylated PCR product} + \text{signal intensity unmethylated PCR product})]$. Methylation status of the samples was assigned unmethylated (U) if $MI < 0.1$, mixed (M/U) if $0.1 < MI < 0.9$ or methylated (M) if $MI > 0.9$.

Additionally, bisulfite sequencing was also performed to further confirm, non-quantitatively, methylation of the AQAMA target sequences for MINT2 and MINT12, as described previously^{7, 36}. Briefly, the sequencing primer sets were designed to flank the region amplified by the AQAMA assay. When it was not possible to design flanking primer sets, either the forward or the reverse AQAMA assay primer was used. The primer sets used for sequencing were: MINT2, 5'-TTTGTAGTTTGTAGTGTGTTTAAATGGAA -3' (forward) and 5'-TCAACACTTTAACAAAATCCAAAATC-3' (reverse), MINT12, 5'- GGGTTTGTAGTTT-GAGGATTAGG-3' (forward) and 5'-CAAAACCATATCTAAATCCTAACCTT-3' (reverse). The amplified PCR product was run on a 2% agarose gel and the single band was confirmed and cut out. DNA was purified from the gel and sequenced with the dye terminator cycle sequencing (DTCS) quick start kit (Beckman Coulter) according to the manufacturer's instructions. Sequencing fragments were analyzed by CAE (Beckman Coulter) and analyzed by the instrument software.

Statistical Analyses

AQAMA assay performance was tested by comparing the linearity of input and measured MI by Pearson's correlation coefficient. We evaluated whether AQAMA can identify marked differences between methylation levels of MINT loci in paired CRC adenoma and cancer cells diverging from normal variance. We calculated Kurtosis of the data distribution. A

positive (>0) Kurtosis denotes that fewer observations cluster near the average and more observations populate the extremes either far above or far below the average compared to the bell curve shape of the normal distribution. To identify outlier values of the measured methylation differences, we performed the Kolmogorov-Smirnov test (KS test). The one-sample KS test compares the empirical distribution function with the cumulative distribution function specified by the null hypothesis (a normal distribution). A significant P -value here indicates that the tested data-set does not adhere to the null hypothesis. Positive and negative extreme differences at which the tested data-set exceeds the normal distribution were calculated.

Results

AQAMA Specificity and Performance

First we evaluated, non-quantitatively, the CpG island methylation status of the MINT loci selected for this study in cancer cell lines. To this end we performed capillary array electrophoresis methylation specific PCR (CAE-MSP) to assess the CpG island methylation status for all 4 selected MINT loci in 5 colorectal and 5 gastric cancer cell lines. These results (**table 1**) were used to identify cell lines that were fully methylated or unmethylated and therefore suitable as template for cloning into vectors, cultured and expanded to be used as template for the AQAMA standards. Before doing so, we further corroborated the methylation status of the CpG islands of two of the MINT loci² (MINT2 and MINT12) in two of the cell lines used for cloning (AGS and FN-0028) by direct bisulfite sequencing. Sequencing confirmed the methylation status as reported by CAE-MSP (see **figure 2a-c** for the sequencing and CAE-MSP results for the MINT12 locus). To gauge the accuracy of AQAMA in assessing various levels of methylation, mixtures of methylated and unmethylated standard cDNA, synthesized from template of which the methylation status was confirmed by at

Cell line	MINT1		MINT2		MINT12		MINT31	
	AQAMA	CAE-MSP	AQAMA	CAE-MSP	AQAMA	CAE-MSP	AQAMA	CAE-MSP
SW480	0.49	M/U	0.47	M/U	0.51	M/U	0.46	M/U
SW620	0.51	M/U	0.53	M/U	0.49	M/U	0.45	M/U
DLD-1	0.49	M/U	1	M	0.48	M/U	0.44	M/U
HT-29	0.50	M/U	1	M	0.48	M/U	0.47	M/U
LoVo	0	U	0.51	M/U	0.50	M/U	0.47	M/U
AGS	0.54	M/U	1	M	1	M	1	M
KATO-III	0.53	M/U	0.49	M/U	1	M	0.48	M/U
SNU-1	0.49	M/U	0.50	M/U	0	U	1	M
RL-0380	0	U	0	U	0	U	0	U
FN-0028	0	U	0	U	0	U	0	U

TABLE 1. Comparison of MI Assessment by AQAMA and CAE-MSP in Cell Lines

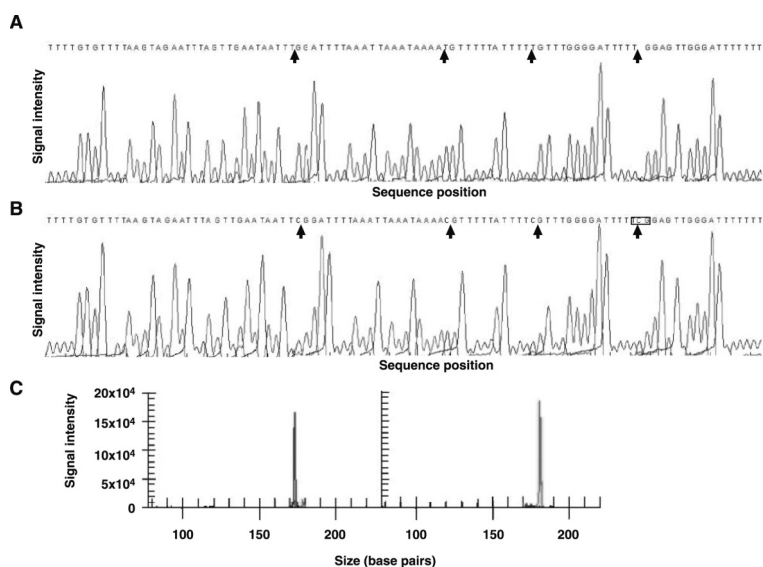


Figure 2. Methylation assessment results for MINT12. Representative direct bisulfite sequencing (forward direction) for FN-0028 (A) and AGS (B). Arrows, CpG sites. Boxed site, hemi-methylation. C. Representative results of CAE detection of labeled (left, methylated; right, unmethylated) products after MSP for FN-0028 and AGS, respectively.

least one technique, were prepared and measured as unknown samples. The mixtures were prepared from the methylated and unmethylated diluted 1×10^3 copy number DNA standard. The AQAMA assay performance for all four MINT loci was assessed. In **figure 3**, the results of two representative independent experiments for each MINT locus assay are shown. Pearson's correlation coefficient for linearity of the methylation percentage of the known mixture with the AQAMA assay outcome MI-value was not lower than 0.966 ($P < 0.001$). SD of all measured MI levels between the two independent experiments did not exceed 0.08 for the four MINT locus assays. We subsequently quantitatively assessed the ten cell lines analyzed with CAE-MSP by AQAMA. The results showed that there was 100% agreement between the methylation categories of CAE-MSP and the quantitative result of AQAMA (**table 1**). The AQAMA result could be compared with the results from the direct bisulfite sequencing and here was good agreement as well. Strikingly, the quantitative results of the cell lines returned by the AQAMA assay appeared to be categorical. Of the 40 datapoints, 10 were $MI=0$, 7 were $MI=1$ and 23 had $0.44 < MI < 0.54$ with an average MI of 0.49. These results suggest that methylation of MINT loci in the studied cell lines is homogenous. An explanation for this observation is that MINT locus methylation occurs at a single or both alleles in cell lines.

CRC tumor-adenoma methylation level differences

To demonstrate the value of assessing primary CRC tissue methylation levels by AQAMA,

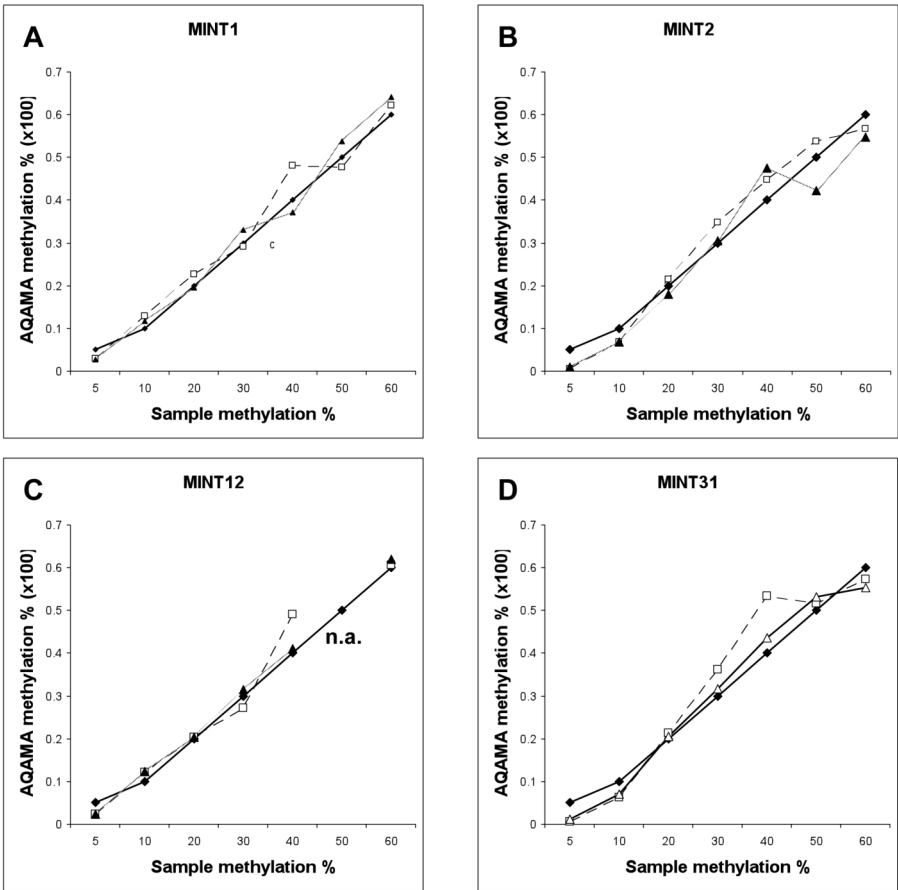


Figure 3. A to D. Graphs representing the correlation between result of two independently assessed AQAMA assay MI levels (Y-axis) and input DNA methylation percentage (X-axis). $\square$ and $\blacktriangle$, results of the two experiments. $\blacklozenge$, expected MI.

we investigated application of the technique and its utility when combined with on-slide SBM. We tested whether the AQAMA assay has the ability to detect differences in methylation levels between pre-malignant and malignant CRC stages. A schematic overview of combining AQAMA with on-slide SBM is given in **figure 4**. Seventy-two cases were selected based on review of histopathology indicating that, along with invasive cancer cells, the specimen also had an area of tissue containing the precursor adenomatous lesion. Each sample was measured in triplicate and the SDs were 0.04, 0.04, 0.05, and 0.06 for MINT1, MINT2, MINT12, and MINT31, respectively. The boxplots of the measured MI values in adenoma and cancer tissue (**figure 5**) clearly show that the MI values are not normally distributed and that samples showing methylation are outliers. The experiments testing the linearity of AQAMA's quantitative qualities showed that AQAMA is able to reliably discri-

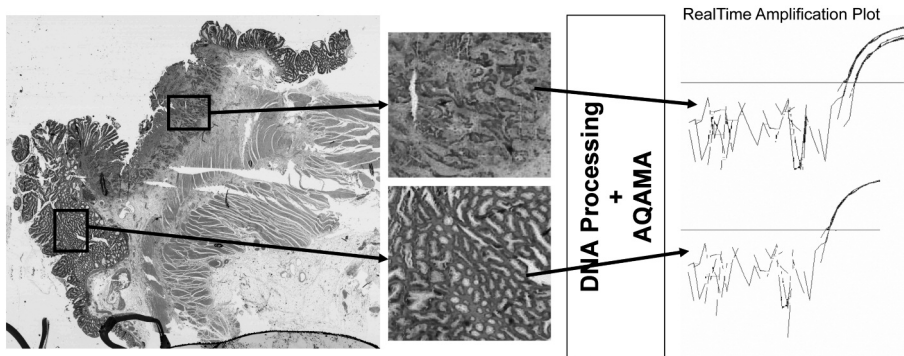


Figure 4. Schematic representation of histology-oriented tissue isolation followed by AQAMA. Left, AQAMA PCR plot. The adenomatous tissue component (bottom marked area) shows only unmethylated fluorescent signal (triplicate results), whereas the cancerous component (top marked area) shows both unmethylated and methylated fluorescent signal. Both signals are visualized here; however, in the raw data analysis, the CT is analyzed separately.

minate a minimum difference of 5% from $MI=0$ or amongst samples. An $MI \geq 0.05$ was detected in 12% versus 11% (MINT1), 35% versus 29% (MINT2), 22% versus 22% (MINT12), and 22% versus 15% (MINT31) in adenoma versus cancer cells, respectively, and none of the proportions differed significantly. It suggests that CRCs with methylation at the MINT loci form subgroups as the majority of CRCs is unmethylated. The total number of MINT loci with $MI \geq 0.05$ per sample did not differ significantly either between adenomas and cancer samples ($P=0.27$). No significant differences in MI value were present between adenomas and cancers with $MI \geq 0.05$ for individual MINT loci. We also analyzed if MI levels differed significantly when methylation at all MINT loci was added up. For this analysis we first included all samples and subsequently only samples with $MI \geq 0.2$ as AQAMA was tested to discriminate 5% difference from zero at a single locus and subsequently 20% from zero at 4 loci. No significant overall event of gain or loss of methylation could be demonstrated in both analyses ($P=0.11$ for all samples, $P=0.20$ for samples with $MI \geq 0.20$) at the combined MINT loci between pre-malignant and malignant CRC lesions. Likely, this is due to the fact that increases as well as decreases in methylation were measured as becomes clear from Figure 5 that shows the measured change in MI level between the 72 adenoma-cancer pairs. Therefore, we also analyzed the distributions of the measured MI changes at individual and at the four combined loci to see whether substantial increases or decreases were measured by AQAMA. Kurtosis is based on the size of a distribution tail. Distributions with relatively large tails are referred to as "leptokurtic" and a distribution with the same kurtosis as the normal distribution is referred to as "mesokurtic" (Figure 6). Kurtosis of the data distributions of the methylation level differences between adenoma and tumor cells was high except for MINT2 (table 2). However, this was still greater than zero and therefore leptokurtic. This suggests that methylation levels in some CRCs change considerably at individual MINT loci and that a global event at multiple loci

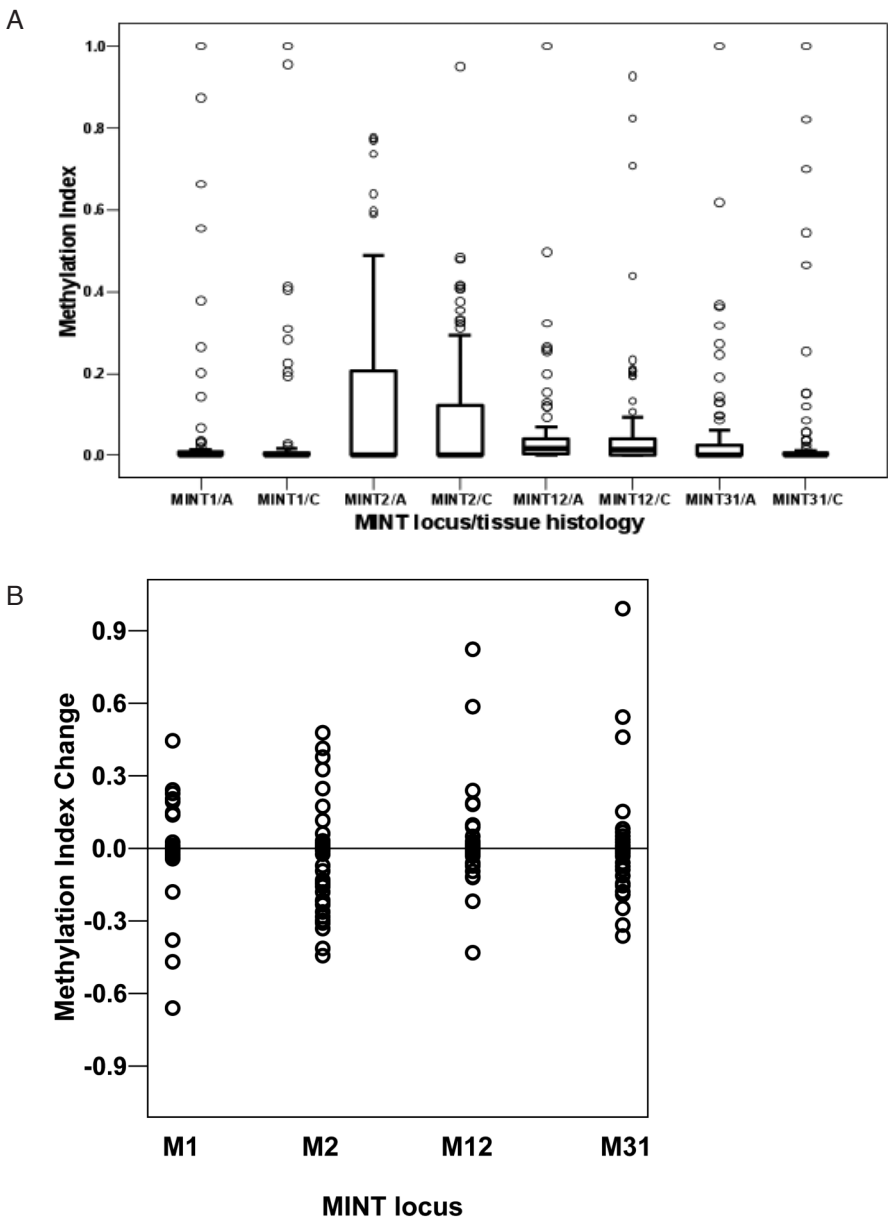


Figure 5. *A.* Boxplots showing the distribution of the measured MI values for each individual MINT locus in adenoma and cancer tissue. *B.* Scatter plot of the measured MI changes detected by the AQAMA assay between adenoma and cancer tissue areas in the same colorectal cancer tissue section for all individual MINT loci. Y-axis, change in MI level calculated as $MI_{cancer} - MI_{adenoma}$.

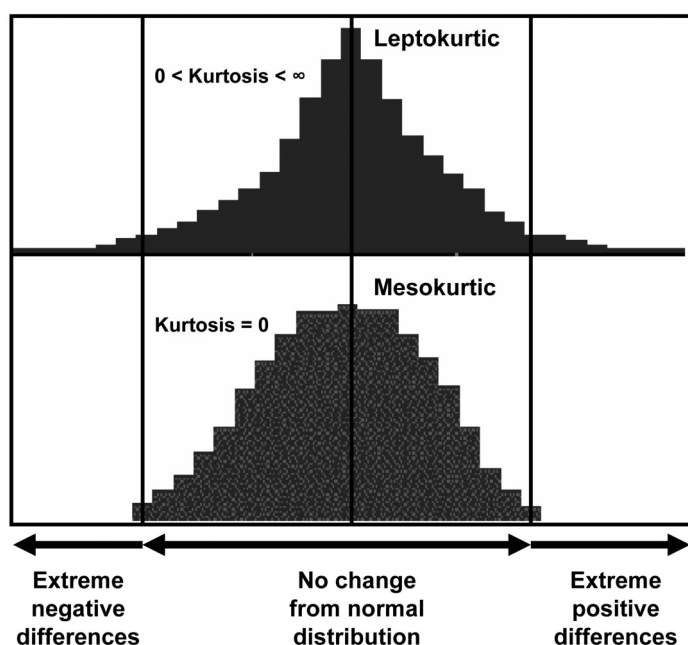


Figure 6. Explanatory representation of leptokurtic and mesokurtic distribution. Vertical bars, cutoff value calculated by Kolmogorov-Smirnov analysis to identify extreme differences between assumed mesokurtic and measured leptokurtic distributions.

Marker	Outlier values*					
	Kurtosis	Median	SD	Negative	Positive	P
MINT1	11.2	-0.006	0.13	-0.36	0.34	<0.001
MINT2	1.9	-0.03	0.16	-0.27	0.24	<0.001
MINT12	17.5	-0.01	0.14	-0.25	0.34	<0.001
MINT31	18.5	-0.001	0.17	-0.25	0.35	<0.001
Total methylation level difference at four MINT loci	15.4	-0.02	0.46	-0.12	0.27	<0.001

TABLE 2. Distribution Characteristics of Individual MINT Locus MI Differences between Paired ColorectalCarcinoma Adenoma and Cancer Cells

*Kolmogorov-Smirnov analysis was used to calculate extreme outlier values of the largest positive and negative points of divergence from the tested normal distribution.

may occur. To identify how many tumors increase or decrease their methylation, we performed one-sample Kolmogorov-Smirnov (KS) analysis on the data distribution of individual MINT loci and overall MINT methylation (**table 2**). The strong significance in KS analysis for individual and total MINT loci shows a clear deviation from the null-hypothesis

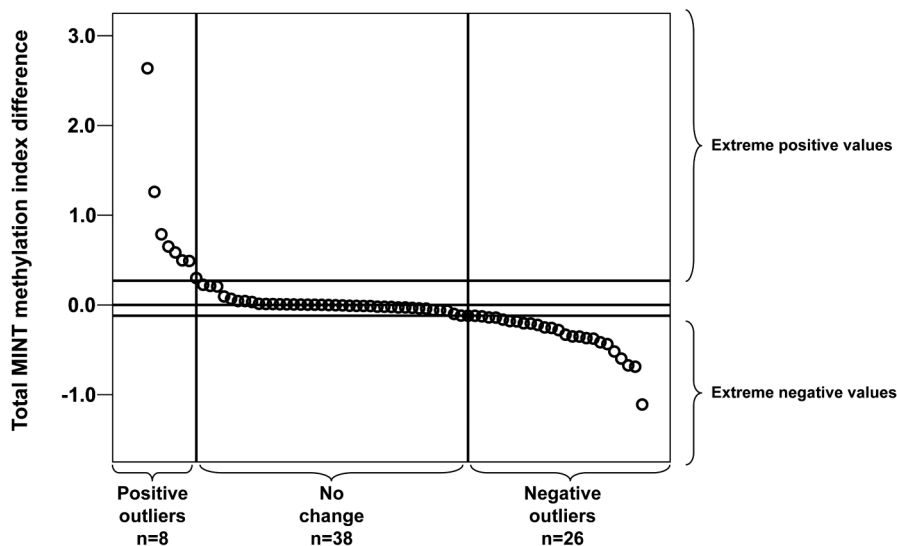


Figure 7. Distribution of values of summed up MINT methylation level differences (Y-axis) between adenoma and cancer tissue from the same patient at MINT1, MINT2, MINT12, and MINT31 assessed by AQAMA. X-axis, different cases (dimensionless). Horizontal reference lines, cutoff values as calculated by Kolmogorov-Smirnov analysis from Table 3. Vertical bars, dividing lines grouping into cases with extreme positive methylation differences, no differences, and extreme negative differences.

(normal distribution), implying that the found positive and negative outlier values do not result from variance by chance. The KS test can calculate the most extreme differences as the largest positive and negative points of divergence between the tested dataset and normal distribution. For total methylation, twenty-six (36%) cases were identified with a decrease in methylation (MI difference <0.12), and 8 (11%) cases were identified with increases (MI difference >0.27). Fifty-three percent of cases had change in methylation levels that did not exceed the tested normal distribution (**figure 7**).

Currently, there is no established technique to adequately confirm the measured differences in MI value between the small areas of paraffin tissue by AQAMA. It has been consistently reported that CRCs with increased methylation are found in the right colon²¹⁻²³. As an external validation, we therefore analyzed whether the positive MI change category identified by the KS-analysis correlated to the location of the tumors in the large bowel (**table 3**). Tumor location did significantly correlate to MI change category ($P=0.03$). Seven of the eight identified cases with an increase in MI were in the right colon. The single positive MI change case that was identified in the rectum was from a 48 year old female with an undifferentiated tumor. Cases with extreme negative MI change were equally distributed over the right and left colon. Sixty-four percent of cases with no change were in the left colon. Additionally we analyzed whether the MI change categories were correlated to age, sex, or tumor differentiation. No associations were seen between the assigned

Site*	Kolmogorov-Smirnov analysis assigned categories			
	Negative outliers	No change	Positive outliers	Total
Right colon				
Cecum	2	7	4	13
Ascending colon	4	2	2	8
Hepatic flexure	4	2	0	6
Transverse colon	4	4	1	9
Left colon				
Splenic flexure	0	0	0	0
Descending colon	1	2	0	3
Sigmoid colon	5	4	0	9
Rectosigmoid	1	5	0	6
Rectum	5	12	1	18
Total	26	38	8	72

TABLE 3. Association between MI Change Category and Tumor Location

*Tumor location significantly associated with MI change category: $P = 0.03$ (Kruskal-Wallis test).

MI change categories and these parameters.

The results indicate that AOAMA can identify CRCs with gains and losses of DNA methylation levels at individual and combined multiple MINT loci between adenomatous dysplastic epithelial cells and invasively growing adenocarcinoma cells. MINT loci 1, 2, 12, and 31 were originally identified to be methylated in CRC and not in normal colorectal epithelial cells. Our study analyzed adenomatous components of existing CRCs with common histopathology, and therefore focuses on sporadic pre-malignant lesions that will develop into cancer. This novel approach quantitatively shows that divergent MINT methylation changes accompany the malignant turning point of CRC subsets.

Discussion

Hypermethylation of CpG islands is an early event in the development of CRC²⁴⁻²⁶. Better identification of methylation changes when adenomatous epithelial cells manifest invasive growth could greatly enhance our knowledge of the malignant turning point. To date, the assessment of confined areas with specific histopathology in PEAT specimens by PCR techniques for methylation status has not been efficient. Relatively large amounts of DNA are required to compensate for the inevitable loss of DNA during the standard protocol for SBM. We have previously employed a model of comparing, by MSP, the methylation status of CRC cells within the same tissue section showing invasion with cells from the adenomatous precursor lesion¹⁴. In this study, we applied an informative, quantitative technique, providing more detailed information about the methylation status than the

dichotomous results of MSP. The absolute quantitative aspect of AQAMA is well suitable to be used in combination with on-slide SBM. The use of a standard curve from serial dilutions of DNA construct allows precise assessment of copy numbers in the sample DNA over a wide range of input concentrations. The original report of AQAMA describes that input DNA before sodium bisulfite treatment was standardized to 1 μ g. The amount of input DNA in the AQAMA assay isolated from 1-2 mm² of tissue of a 7 μ m section is difficult to standardize. Therefore the control on the linearity of the PCR reaction that the standard curve provides over a wide range of input concentrations complies well with on-slide SBM. Also, standardization allows comparison of results amongst different PCR runs. We demonstrated that levels of MINT locus methylation in CRCs can accumulate at multiple genomic CpG island loci (predominantly in the right colon), but can also decrease during malignant change in CRC. It is interesting to note that there seems to be no uniform event that most CRCs undergo during malignant transformation for the methylation at MINT loci. In addition, the data indicate that subgroups of CRCs may exist that can lose, stabilize or gain methylation in the gene promoter region. Considering the silencing effect of methylation, the divergent development of methylation patterns could lead to differential gene expression signatures proven to be clinically relevant in CRC^{27, 28}.

The capacity of AQAMA to discern differences in methylation levels was excellent as it was measured in a range of MI = 0.05 through MI=0.6 with increments of 0.1. However, it was noted that the accuracy decreases at methylation levels containing a MI < 0.05. Single reaction AQAMA, therefore, is likely to have less value in picking up the so-called “needle in a hay stack” from a large population of normal cells, as in micrometastatic tumor cells of CRC in lymph nodes. To use AQAMA for such purposes of detection, PCR reactions with methylated and unmethylated probes may be run separately.

On-slide SBM reduces the risk of non-cancer cell contamination compared to DNA isolated from whole PEAT sections, where normal colon tissue areas, such as muscle layers and serosal layers, are usually present. Because the studied tissue area can be confined to a specific 1-2 mm² tumor sample, the DNA source is usually more homogeneous, resulting in a more reliable representation of the amount of methylated alleles in the tumor. Another important aspect in measurement of DNA methylation levels is that human error and inter-assay variability is kept to a minimum. The control that the single reaction AQAMA assay provides is that results can be analyzed directly without the need to compensate for the variability of two or three separate PCR reactions with different settings and reaction kinetics^{29, 30}.

In summary, AQAMA is a very sensitive assay that can reliably detect 10% differences in methylation between samples. It utilizes a real-time PCR technique with reported robustness and reproducibility³¹. The single-reaction assay makes AQAMA suitable for the assessment of large clinical sample sizes, as required in biomarker studies. The technique can be applied to widely accessible PEAT specimens, and uses a minimal amount of tissue (one single 7 μ m section), making it suitable for retrospective analysis. We demonstrated that, when combined with on-slide SBM, AQAMA forms a useful assay that can give new insights in the development of epigenetic patterns during colorectal carcinogenesis using archival paraffin-embedded specimens.

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CHAPTER 4

LINE-1 methylation analysis in colorectal cancer shows progression of hypomethylation at early stage

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Abstract

Background: Methylation levels of DNA repeats such as long interspersed nucleotide elements (LINE-1) are representative of global methylation status and play an important role in maintenance of genomic stability. LINE-1 methylation is known to be disrupted in many types of cancer. In this study we report on high detail analyses of LINE-1 methylation levels at different stages of colorectal cancer (CRC) development and at different stage of CRC disease.

Material and Methods: Sample DNA was collected by laser-capture microdissection (LCM) treated by a newly introduced protocol of on-cap (*in situ*) DNA sodium bisulfite modification (SBM). LINE-1 hypomethylation index (LHI), a higher index indicates more hypomethylation, was measured by absolute quantitative analysis of methylated alleles (AQAMA).

Results: LHI was significantly higher in adenoma tissue (n=25) compared to normal epithelium (n=23, $P=0.0003$) and cancer mesenchymal tissue (n=22, $P<0.0001$). LHI did not differ significantly between adenoma and cancer tissue (n=24, $P=0.20$). No significant differences were found in LHI between normal epithelium from non-cancer (n=13), adenoma (n=18) and cancer patients. Tumor heterogeneity was tested and LHI did not differ significantly comparing cells collected from a tumor's luminal side compared with cells from the invasive margin. LHI increased with higher T-stage ($P<0.04$) and was significantly higher in node-positive versus node-negative disease ($P=0.03$) and was significantly higher in stage IV versus all other disease stages ($P<0.05$).

Conclusion: By using quantitative LINE-1 analysis with on-cap SBM microdissection techniques this is the first study to demonstrate hypomethylation of LINE-1 to increase with advanced disease stage in colorectal cancer.

Introduction

LINE-1 stands for long interspersed nucleotide elements and constitutes about 17-18% of the human genome. Roughly 500,000 truncated and 3000 to 5000 full length LINE-1s are dispersed throughout the human genome¹. LINE-1 sequences are moderately CpG rich and most CpG's locate in the 5' region that can behave as a internal promoter². In normal somatic cells, LINE-1s are heavily methylated restricting activities of retrotransposal elements and genomic instability^{2, 3}.

Numerous studies have been reported identifying colorectal cancer (CRC) cancer-associated epigenetic aberrations such as promoter region hypermethylation of tumor suppressor genes⁴. Another characteristic alteration in CRC regarding DNA methylation in cancer is global hypomethylation^{5, 6}. LINE-1 methylation status is thought to represent the genome-wide DNA methylation status such as global hypomethylation, since LINE-1 sequences are highly repeated and widely interspersed human retrotransposons. The role of global DNA hypomethylation in cancer is unclear, although it may cause genomic instability and correlates with tumor progression and invasiveness in several types of cancer including colon cancer. Regarding colon cancer, Bariol et al. revealed that the global DNA methylation level was higher in neoplastic lesions (including hyperplastic polyps and adenoma) than in normal mucosa⁷. Ogino et al. suggests that LINE-1 hypomethylation status of colon cancer associates with poor prognosis⁸. The progression of LINE-1 hypomethylation level according to the sequence of colorectal tumor disease stage remains unclear to date.

Cancer tissue is highly heterogeneous and contains various cells, such as normal (i.e. stromal cells and infiltrating lymphocytes) and malignant cells and their distribution differs in each cancer patient. This heterogeneous nature of cancer tissue may confound molecular analysis, especially in case of a molecular target such as LINE-1 methylation analysis, because unmethylated LINE-1 is non-cancer-specific. In this study we therefore utilize automated laser-capture microdissection (LCM) to harvest the cells of interest directly without contamination of non-cancer cells. A precise quantification method for analysis of methylation status is further necessary to evaluate the degree of LINE-1 methylation. Absolute quantitative assessment of methylated alleles (AQAMA) assays was used for this purpose in this study. As previously demonstrated, the capacity of AQAMA to discern differences in methylation level was excellent⁹. The single reaction AQAMA assay provides good control because results can be analyzed without the need to compensate for the variability of two or three separate PCR reactions with different setting and reaction kinetics. The approach allows accurate derived analysis of histopathology microdissected cells from PE tissue sections.

Sodium bisulfite modification (SBM) of genomic DNA is commonly used method to discern methylation status of CpG islands¹⁰. Sodium bisulfite converts cytosines but not 5-methylcytosines to uracil and the changes in sequence of the DNA after SBM can be analyzed by many kinds of methylation assays including AQAMA assay. Conventional SBM results into 84% to 96% loss of sample DNA¹¹. This significant loss of template DNA causes the necessity for a high volume of start material tissue samples and high number of cycles for PCR. To reduce the loss of sample DNA from cell collected by LCM we developed on-cap SBM. On-cap SBM was designed to minimize DNA loss by eliminating DNA purification steps. In the process of on-cap SBM, DNA is modified *in-situ* while the cells are

still attached on the cap which is used for LCM. DNA is collected afterwards and processed for AQAMA. We have developed and optimized a protocol for on-cap SBM in this study by which sufficient DNA conversion rates can be achieved and relatively low volumes of tissue sample are needed for the subsequent AQAMA LINE-1 hypomethylation assay.

LCM, on-cap SBM and AQAMA was combined and used in samples from patients with adenomas containing invasive adenocarcinoma and patients with advanced colon cancer. This allowed to analyze the development of LINE-1 hypomethylation levels during tumor progression and differences of LINE-1 hypomethylation levels between node positive and node negative colon cancer patients. Our results indicate that LINE-1 hypomethylation occurs in the early stage of colorectal tumorigenesis such as adenoma and proceeds according to the tumor stage such as depth of invasion and establishment of lymph node metastasis.

Materials and Methods

Measurement of LINE1 hypomethylation

The methylation status of LINE1 was evaluated by absolute quantitative assessment of methylation alleles (AQAMA) assay to make accurate assessment of CpG island methylation levels⁹. Before performing the AQAMA assay of LINE1 methylation status, sodium bisulfite modification (SBM) was applied on each DNA sample as previously described¹². AQAMA requires one forward and one reverse primer which will amplify the target sequence independent from the methylation status, as those forward and reverse primer set does not contain any CpG. The methylation status is assessed by two minor-groove-binding (MGB) molecule containing probes (Applied Biosystems): one methylation specific and one unmethylation specific. The 5' primer, 3' primer, methylation-specific probe and unmethylation-specific probe are listed as follows: 5'-GGGTTTATTTATTAGGGAGTGTTAGA-3' (forward), 5'-TCACCCCTTTCTTTAACTCAAA-3' (reverse), FAM-5'-TGCGCGAGTC-GAAGT-3'-MGB-BHQ and VIC-5'-TGTGTGAGTTGAAGTAGGG-3'-MGB-BHQ. The reaction mixture for each AQAMA PCR consisted of DNA template, 0.4 µmol/L of the forward and reverse primer, 1.4 unit of iTaq DNA polymerase (Bio-Rad, Hercules, CA, USA), 350 µmol/L of each deoxynucleotide triphosphate and 0.025 pmol of each MGB probe with 5 mmol/L Mg²⁺. PCR amplification was performed with pre-cycle heat activation of DNA polymerase at 95° C for 10 minutes, followed by 40 cycles of denaturation at 95° C for 15 seconds, annealing and extension at 60° C for 60 seconds. The absolute copy number in each sample was determined using a standard curve established by amplifying six aliquot duplicates of templates with known copy numbers (10⁶ to 10¹ copies). All quantitative PCR assays were performed in a blinded fashion without knowledge of specimen identity. Mean values were calculated from triplicate reactions. LINE1 hypomethylation index (LHI) of each sample was calculated as follows: LINE1 LHI = unmethylated copy number / (methylated copy number + unmethylated copy number).

On-cap SBM assay optimization

To measure LINE1 hypomethylation index after micro-dissection using LCM, we develo-

ped the on-cap SBM procedure based on previously introduced on-slide SBM technique. On-cap SBM was designed to analyze methylation status of genes in small amount of DNA obtained from formalin fixed paraffin embedded archival tissue (FF-PEAT). On-cap SBM aims to eliminate DNA isolation and purification steps that are performed before the actual SBM in the classic procedure which is the main cause of DNA loss. On-cap SBM contains three steps; denaturing of DNA, SBM and collection of modified DNA. First, the cells micro-dissected from deparaffinized and rehydrated tissue sections that stick on the cap used in LCM are incubated in 0.2 mol/L NaOH at room temperature for 15 min. Then the samples on the cap are incubated in 3 mol/L sodium bisulfite solution with 0.5 mmol/L hydroquinone (pH 5) in the dark. Three incubation setting were tested regarding the conversion rates (the rate of modification of cytosine to uracil) and yields of modified DNA and incubation setting of 60 degrees for 8 hours was chosen as optimal (see results section). After incubation, samples on the cap were rinsed with distilled water, soaked in 0.3 mol/L NaOH for 15 minutes to desulfonate the modified cytosines, and then desalted in distilled water at 60°C for 2 hours. Finally, 50 µL lysis buffer containing 4 µg proteinase K, 2.5% Tween 20, 50 mmol/L Tris, and 1 mmol/L EDTA were added on the cap and incubated at 50°C for 24 hours followed by heat deactivation of proteinase K at 95°C for 10 minutes. In each subsequent AQAMA reaction, 1-2 µL lysate was used as a template without DNA purification.

FF-PEAT colorectal tumor samples

FF-PEAT samples of colorectal cancers were obtained from patients who underwent colectomy or proctectomy between 1995 and 1998 at Saint John's Health Center (Santa Monica, CA) and consented for research use of their tissue specimens according to the guidelines of Saint John's Health Center/John Wayne Cancer Institute's (Santa Monica, CA) review board. All tissue specimens had been fixed in 10% buffered formalin for 24 hours and paraffin embedded. For LCM followed by on-cap SBM and LINE1 methylation analysis by AQAMA, sections (4 µm) were cut with a microtome from each FF-PEAT block.

To assess the differences of LINE-1 methylation status between normal mucosa, adenoma, cancer and cancer mesenchymal connective tissue, 25 samples of early CRC in adenoma were selected by a pathologist specialized in colorectal cancer that contained all of these four tissue categories. To study malignant alteration, 92 surgically resected specimen CRC were collected to investigate the alteration of LINE1 methylation status in accordance with cancer progression. The correlation between clinico-pathological stage such as T stage, nodal status and dukes stage and LINE-1 methylation index were analyzed.

Statistical analysis

All data are expressed as mean+/-S.D., and percentages as appropriate. Mean value of LHI between two groups such as adenoma and early cancer, node negative and node positive were compared using Student's *t*-test. All statistical analysis including receiver operating characteristic (ROC) curve analysis were performed using JMP 8 (SAS Institute, Cary, NC) and a *P*-value less than 0.05 (two-sided) was considered significant.

Results

AQAMA linearity for LINE-1 methylation level assessment

First, we evaluated the accuracy of AQAMA in assessing various levels of LINE-1 methylation. For this study we prepared stepwise mixtures of the methylated and unmethylated cDNA standard and measured them as samples with unknown methylation level. The linearity of AQAMA assay for LINE-1 methylation level (**figure 1**) was evaluated by Pearson's coefficient of linearity: 0.9897 ($P < 0.001$). This results shows that the AQAMA LINE-1 assay can discern 10% methylation level differences adequately.

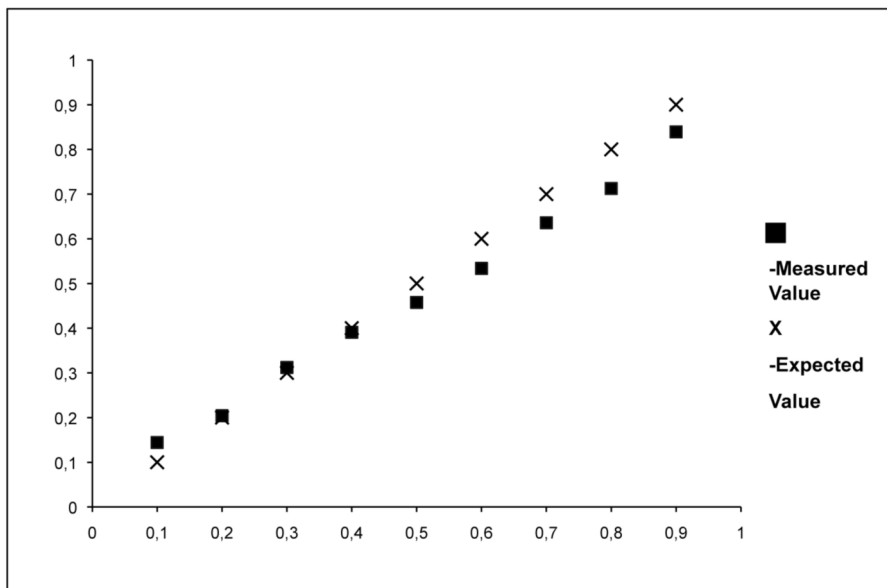


Figure 1. Graph showing linearity of the AQAMA LINE-1 methylation assay to measure 10% differences in sample hypomethylation levels.

Optimization of on-cap SBM settings

Automated LCM was performed to harvest cells of interest (**figure 2a**) to establish how many cells are needed for successful AQAMA measurements. Three different sample volumes from tissue areas of 10^4 , 10^5 and $10^6 \mu\text{m}^2$ harvested from $7\mu\text{m}$ thickness formalin fixed paraffin embedded archived tissue (FF-PEAT) were tested. AQAMA measures copy numbers and we established that $2 \times 10^5 \mu\text{m}^2$ of $7\mu\text{m}$ thickness FF-PEAT results in about 1×10^5 copy numbers of modified LINE-1 DNA and this provides clearly detectable fluorescent probe amplification signals for the LINE-1 methylation assay. After LCM, on-cap SBM was optimized in a similar manner as was performed in the reported studies introducing on-slide SBM¹³. The conversion rate and yield were evaluated using eight tissue samples by assessing the copy number of modified and unmodified ALU repeat sequences, as previ-

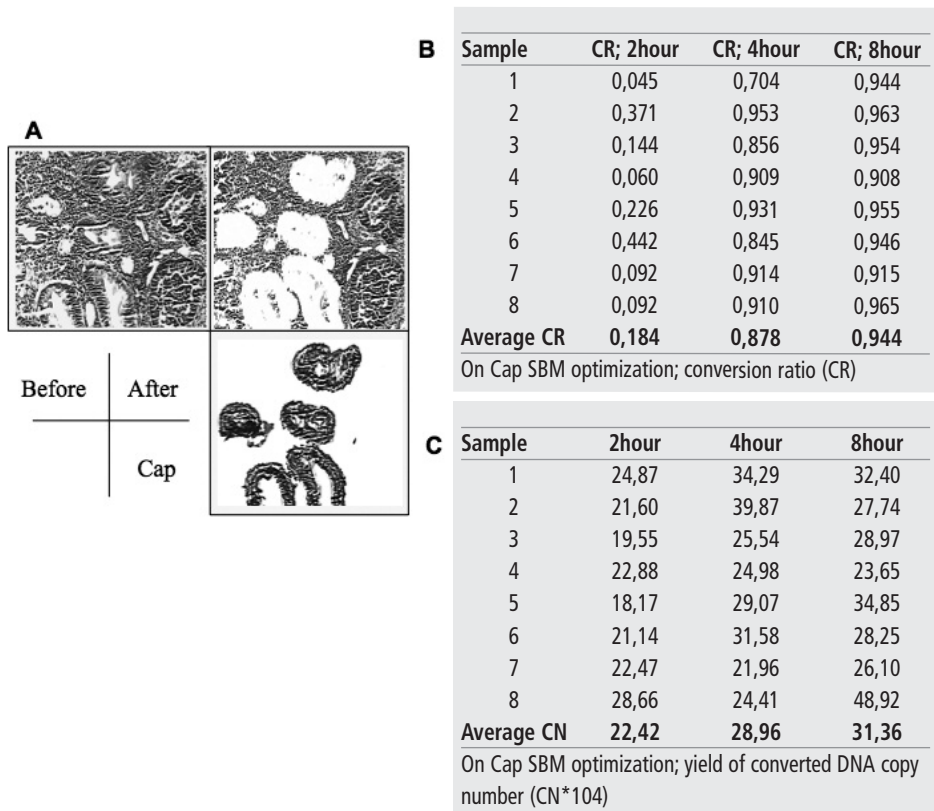


Figure 2. Representations of LCM combined with on-cap SBM optimization studies. In **A**, showing specific pick-up of target cells by automated LCM. In **B** and **C**, showing results for DNA conversion rate (%) and for DNA content ($\times 10^4$ copy numbers), respectively, in 8 different samples at 3 different incubation periods.

sly published¹³. The conversion rates of modified DNA by on-cap SBM were $18.4 \pm 14.9\%$, $87.8 \pm 7.8\%$ and $94.4 \pm 2.1\%$ at incubation setting of 60°C for 2, 4 and 8 hrs, respectively (**figure 2b**). The conversion rates increased with increasing duration of incubation and after 8 hours it reached about 95%. The yield of total DNA (modified and unmodified DNA) did not decrease with increasing incubation time up to 8 (**figure 2c**). From these results, incubation setting of 60 for 8 hrs was chosen as optimal for on-cap SBM.

LINE-1 hypomethylation levels in colorectal tumor progression and cancer mesenchyme.

We have selected 25 patients with colorectal cancer in adenoma. Using LCM, four different tissue samples (normal mucosa, adenoma, cancer and cancer mesenchymal tissue) were collected from each patient. On-cap SBM and LINE-1 AQAMA assay was performed on each sample. Level of LINE-1 hypomethylation (LHI) were calculated as $Q_{\text{unmeth}} / (Q_{\text{unmeth}} + Q_{\text{meth}})$, where Q_{unmeth} and Q_{meth} are the absolute copy numbers of unmethyla-

ted and methylated LINE-1, respectively. In normal mucosa adjacent to tumor lesions and cancer mesenchyme, the average LHI was 0.382 and 0.366, respectively. There was no difference of LHI between cancer mesenchyme and normal mucosa adjacent to tumor. LHI was significantly higher in adenoma part and cancer part than in normal mucosa (mean LHI=0.486, 0.523 and 0.382, respectively) (**figure 3**). From this we concluded that LINE-1 hypomethylation occurs in the early period of colorectal tumorigenesis. Further, these experiments demonstrated the necessity of using detailed procedures for sample DNA collection when analyzing LINE-1 methylation as methylation levels of cancer mesenchyme varied widely and are therefore likely to influence results when LCM is not used.

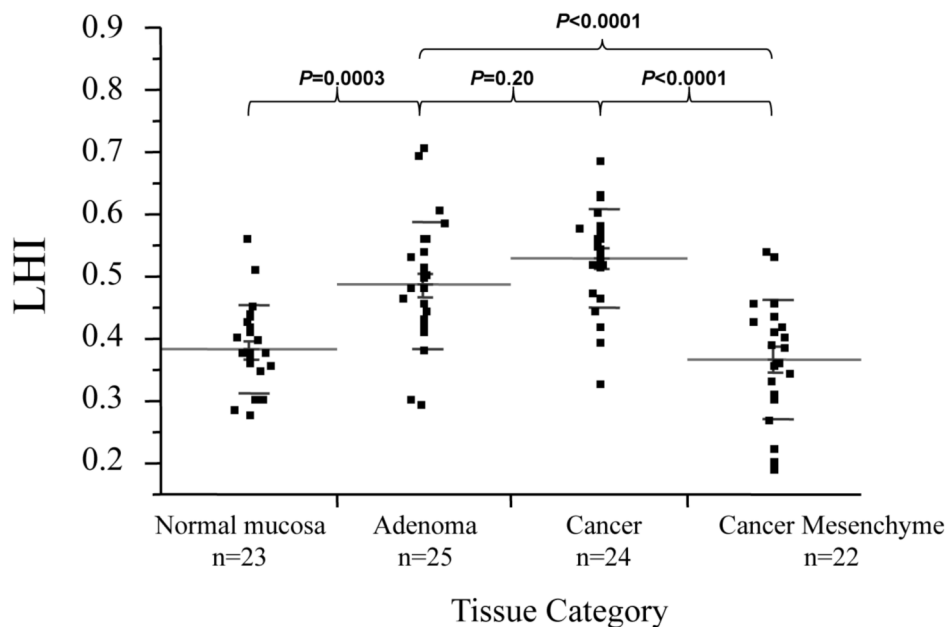


Figure 3. LHI assessment in normal large bowel epithelium, adenomatous cells, cancer cells and CRC mesenchymal cells.

LINE-1 hypomethylation tissue heterogeneity

To test whether there is heterogeneity of LINE-1 hypomethylation within a tumor, twenty T3N0 tumors were selected. Sample DNA was collected from the luminal surface and the deepest invasional site of each tumor. LHI of those two loci were compared (**figure 4**). The mean LHI was 0.58 in the surface and 0.54 in the deepest loci, respectively. LHIs were similar in the surface and in the deepest loci and tumor heterogeneity of LINE-1 hypomethylation could not be observed.

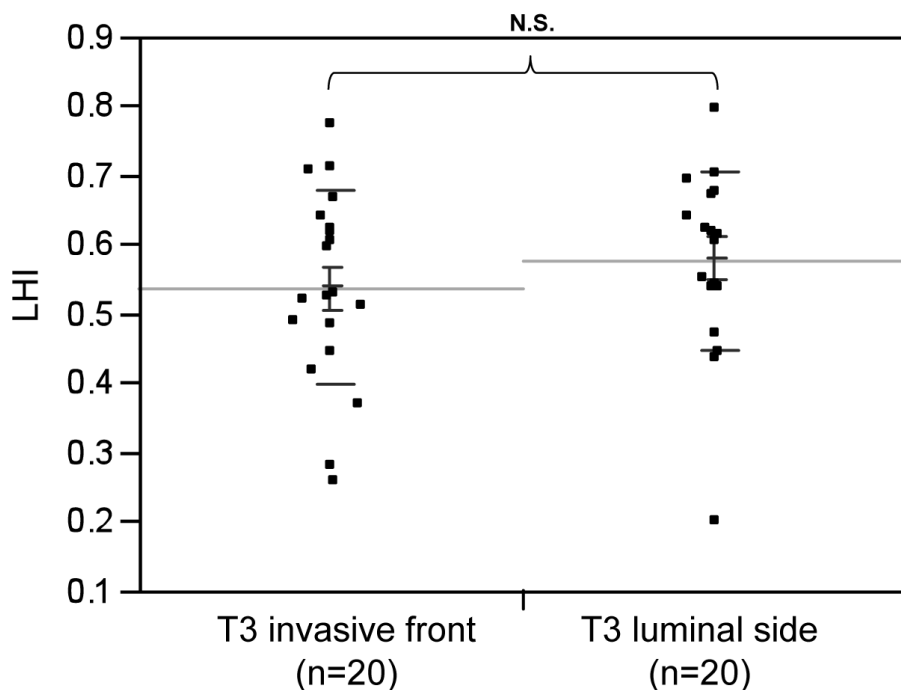


Figure 4. Measured LHI values in T3N0 CRC tumors comparing cells obtained from the surface luminal tumor area to the deep invasional tumor area.

LINE-1 hypomethylation and CRC stage

To assess the alteration of LINE-1 hypomethylation level according to tumor progression, the correlation between Dukes stage and LHI was analyzed. Duke A samples (n=38), Dukes B (n=18) and Dukes C (n=29) samples were selected (**figure 5a**). The mean LHI in Dukes A, B and C was 0.533, 0.607 and 0.621, respectively. Dukes B and C tumors showed significantly higher levels of LINE-1 hypomethylation than Dukes A tumors. T1 (n=24), T2 (n=21), T3 (n=21) and T4 (n=26) cases were assessed, in which 35 positive lymph node cases were included. The increase of the LINE-1 hypomethylation level according to tumor invasion (**figure 5b**) and the difference of LINE-1 hypomethylation between lymph node positive and negative cases were assessed (**figure 5c**). The mean LHI of T1, T2, T3 and T4 was 0.50, 0.58, 0.60 and 0.65, respectively. Cases with positive lymph nodes showed significantly ($P=0.03$) more LINE-1 hypomethylation (LHI=0.64) than cases with negative lymph nodes (LHI=0.54). T stage advancement correlated significantly with increasing LHI ($p=0.01$). LINE-1 hypomethylation levels differed significantly between node positive and node negative cancers and the AUC was 0.69 to predict node positive cancer from whole samples according to ROC curve analysis (**figure 5d**).

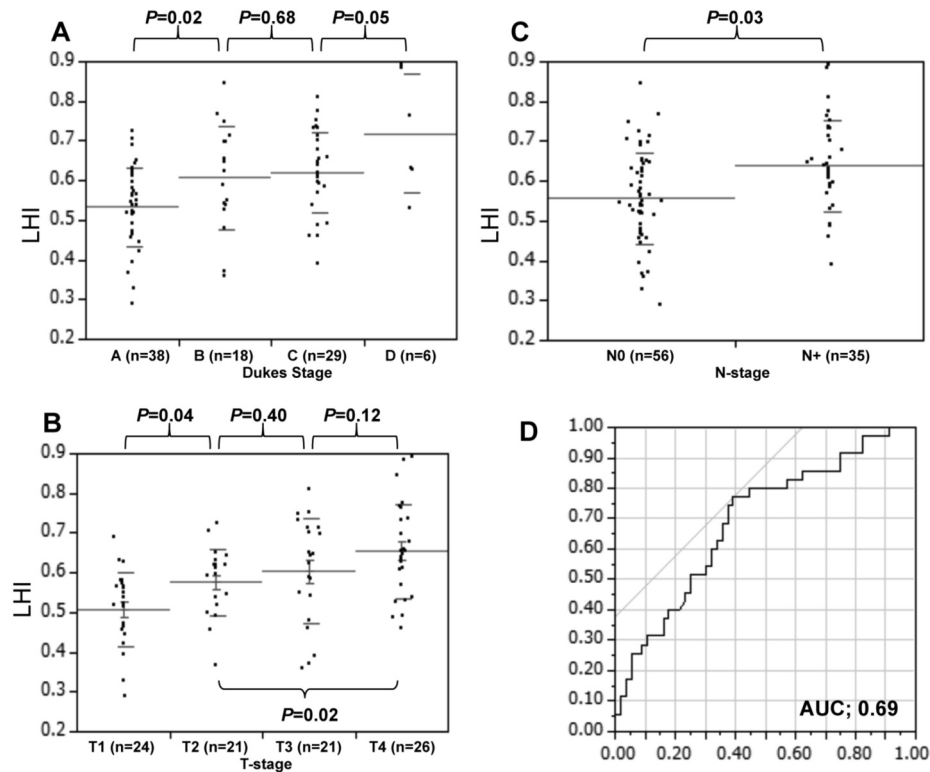


Figure 5. Measured LHI values comparisons between clinical disease stage classifications. In **A**, **B** and **C** differences are shown between Duke stage, T-stage and N-stage, respectively. In **D**, a receiver-operating curve (ROC) analysis is given for prediction of lymph node status by primary tumor LINE-1 hypomethylation analysis with an area under the curve (AUC) of 0.69.

Discussion

The alteration of DNA methylation patterns has been studied in many types of cancer¹⁴. Neoplasia is now epigenetically characterized by hypermethylation of specific cancer associated gene promoter regions and global DNA hypomethylation^{15, 16}. LINE-1 constitutes about 17-18% of the human genome and about 500,000 truncated and 3000-5000 full length LINE-1s are dispersed throughout the human genome^{1, 17}. LINE-1 sequences are moderately CpG rich and most CpG locate in the 5' region that can behave as an internal promoter. Therefore LINE-1 hypomethylation has been used as a surrogate for global DNA hypomethylation. LINE-1 hypomethylation in colorectal cancer has been described in several aspects; hypomethylation in cancer and adjacent normal mucosa, inverse association with microsatellite instability and association with poor prognosis^{8, 18-20}.

LINE-1 hypomethylation is not specific for cancer cells and about one third of LINE-1 is unmethylated in normal mucosa and cancer mesenchymal cells^{21, 22}. The possible contamination of sample DNA considered to be cancer with mesenchymal cells such as fibroblasts or lymphocytes may therefore cause inaccurate assessment. For this reason, especially regarding LINE-1 methylation analysis, isolation of target cells is necessary. To minimize the contamination and obtain to cells of interest only, we successfully integrated automated LCM in LINE-1 methylation analyses this study which has not been reported to date. We introduce on-cap SBM as a novel approach which makes DNA methylation assay efficient and enables evaluation of cells from small areas of target regions.

On-cap SBM was designed based on on-slide SBM¹³. On-slide SBM is a powerful procedure for the methylation analysis using FF-PEAT, however, after on-slide SBM the specimen becomes too adhesive to the glass slide making efficient LCM to capture target cells difficult at times. On-cap SBM eliminates the numerous DNA isolation and purification steps of classic SBM which are the main cause of DNA loss dealing with small amounts of DNA such as isolated by LCM or from serum. We performed optimization studies and an 8 hour incubation showed excellent DNA conversion rates of approximately 95% was achieved and this relatively long incubation duration did not result into loss of converted DNA.

Combining automated LCM, on-cap SBM and AQAMA assay, LINE-1 hypomethylation was demonstrated in adenoma, an early stage of colorectal tumorigenesis, which is in agreement with previous studies^{8, 20}. Furthermore, the level of hypomethylation increased according to tumor stage. In previous studies, no correlation between tumor stage in colorectal cancer and LINE-1 hypomethylation level has been reported. One of the reasons for those differences may be the degree of mesenchymal cell DNA contamination to the sample DNA. Our results demonstrate that not only adjacent normal mucosa but also mesenchymal cells in cancer shows about one third loss of LINE-1 hypomethylation compared to normal epithelium. Sample DNA contamination with those cells causes decrease of LINE-1 hypomethylation level and may result in failure to detect the differences between early and advanced cancer.

Our method enabled methylation analysis of sample DNA from small areas about $2 \times 10^5 \mu\text{m}^2$ and this enabled to analyze heterogeneity of LINE-1 methylation status within the tumor. The comparison between the deepest part and the surface of the tumor revealed that tumor heterogeneity of LINE-1 methylation status is relatively subtle. The results suggest that colorectal tumor biopsy specimens collected during colonoscopy are representative can be used for a tumor's LINE-1 methylation status analysis. LINE-1 hypomethylation levels differed significantly between node positive and node negative cancers and the AUC was 0.69 to predict node positive cancer from whole samples according to the ROC curve analyses. The LINE-1 unmethylation index of colorectal tumor biopsy sample may therefore be useful to preoperatively predict lymph node status of colon cancer. Prospective studies are needed to confirm our study's findings.

In conclusion our study demonstrated that LINE-1 methylation status correlates with clinical stage of colon cancer and its accurate analysis with LCM or equivalent techniques is mandatory. The combination of LCM, on-cap SBM and AQAMA is a powerful method for such accurate analysis.

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Section 2:

DNA methylation in gastrointestinal adenocarcinoma

CHAPTER 5

Epigenetic Silencing of Cyclooxygenase-2 Affects Clinical Outcome in Gastric Cancer

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Abstract

Purpose: Overexpression of cyclooxygenase-2 (*COX-2*) in gastric cancer has been shown to enhance tumor progression. We investigated whether silencing by promoter region hypermethylation of the *COX-2* gene contributes to disease outcome in gastric cancer.

Materials and Methods: *COX-2* methylation status was initially assessed by capillary array electrophoresis methylation-specific PCR (CAE-MSP) and *COX-2* protein expression by immunohistochemistry (IHC) in 40 primary gastric cancer tissues in a pilot study. Prognostic endpoints of correlative studies of *COX-2* methylation status were time to recurrence, overall survival, and standard clinicopathologic features. CAE-MSP analysis was then validated in a second independent gastric cancer population (n=137).

Results: *COX-2* methylation was detected in 23% and 28% of the pilot and validation patient groups, respectively. *COX-2* expression (IHC) in gastric tumors inversely correlated with *COX-2* gene methylation status in the pilot study (P=0.02). *COX-2* methylation in tumors was significantly associated with lower T-, N-, and TNM-stage in the validation patient group (P=0.02, P=0.006 and P=0.008, respectively). Patients with *COX-2* methylated tumors had significantly longer time to recurrence (TTR) and improved overall survival (OS) in a multivariate analysis in the smaller (HR=0.08; 95%CI, 0.01-0.65 and HR=0.37; 95%CI, 0.14-1.00, respectively) and larger patient groups (HR=0.49; 95%CI, 0.24-0.99 and HR=0.62; 95%CI, 0.38-0.99, respectively).

Conclusion: Hypermethylation of *COX-2* gene promoter was identified as an independent prognostic factor in gastric cancer patients. The results suggest promoter hypermethylation to be an important regulatory mechanism of *COX-2* expression in gastric cancer and show a beneficiary effect of tumor-related methylation on disease outcome in gastric cancer patients.

Introduction

Despite its decreased incidence, gastric cancer remains the second most common cause of cancer death and the third most common cancer worldwide¹. Recently, gastric cancer therapy has received more attention, since neoadjuvant modalities have shown to improve outcome for resectable tumors²⁻⁴. Molecular surrogate marker(s) of disease outcome could be of benefit in the management of gastric cancer patient treatment.

About 60% of human genes are associated with clusters of CpG dinucleotides, referred to as CpG islands⁵. Clustered methylation of CpG islands at a gene promoter or transcription start site is associated with gene silencing. This epigenetic event has been observed in many genes of different cancers⁶. Hypermethylation of tumor-related regulatory genes may play a significant role in tumor transformation and progression, impacting the clinical course of disease. Recent studies focus on hypermethylation of specific tumor-related genes with suppressing capacities on growth and proliferation of gastric cancer⁷⁻¹¹. Genes regulated by methylation status can have tumor suppressor functions, as well as tumor-inducing capacities, significantly altered. Therefore, gene inactivation by hypermethylation may have dual effects on tumorigenesis and tumor progression. Epigenetic inactivation of genes related with tumor progression has not been well studied in gastric cancer as related to disease outcome.

COX-2 (cyclooxygenase-2/*PTGS2*, prostaglandin-endoperoxide synthetase-2) expression is upregulated in gastrointestinal cancers¹²⁻¹⁵. In gastric cancer, *COX-2* expression is involved in several important tumor progression-related mechanisms, such as angiogenesis¹⁶, inhibition of apoptosis¹⁷, and invasiveness¹⁸. Song et al. demonstrated regulation of *COX-2* mRNA and protein expression by hypermethylation of the *COX-2* promoter region in gastric cancer lines¹⁹. Most gastric cancers overexpress *COX-2*, and, recently, *COX-2* expression assessed by immunohistochemistry (IHC) was identified to impact disease survival²⁰. Because of the reported epigenetic regulation and predictive value of *COX-2* expression, we hypothesized a role for *COX-2* promoter hypermethylation status in the clinical outcome of patients with gastric cancer. To study this, we first assessed *COX-2* promoter methylation status by quantitative methylation-specific PCR (MSP), as well as its relation to *COX-2* protein expression in paraffin-embedded archival tumor (PEAT) specimens of gastric cancer patients with known disease outcome in a pilot study. All patients were enrolled in a randomized, multi-center trial for primary gastric cancer comparing preoperative chemotherapy versus surgery alone^{21,22}. The clinical impact of *COX-2* methylation in the cancer trial patients was studied by correlating disease outcome to tumor *COX-2* methylation status. The MSP findings were then confirmed in a larger, independent validation patient group, selected from another multi-center randomized trial comparing primary tumor resection with limited versus extended nodal dissection^{23,24}.

Materials and Methods

Tumor specimens

The pilot study group contained patients (n=59) accrued in the FAMTX (5-fluorouracil,

doxorubicin, and methotrexate) trial conducted by the Dutch Gastric Cancer Group (DGCC)^{21,22} evaluating preoperative chemotherapy with FAMTX for gastric cancer. As a validation set, patients were used from the D1D2 trial by the DGCC^{23,24}. The trial evaluated (sub)total gastrectomy for gastric cancer with D1 to D2 lymph node dissection of which the latter, included partial removal of spleen and pancreas. All tumors were classified and staged according to the revised guidelines set by the International Union Against Cancer (UICC). PEAT specimens of the primary tumor were collected from patients from both trials. PEAT specimens from gastric tissue biopsies for benign conditions as controls were also collected from 18 patients without a history of malignancy. The protocol for this study was approved by the Human Subjects Institutional Review Boards of both participating institutions (Saint John's Health Center / JWCI; Leiden University Medical Center).

Tissue and DNA preparation

Serial sections from each PEAT specimen were cut. One section (4 μ m) was stained by hematoxylin and eosin and a tumor representative tissue was marked by an expert surgical pathologist for gastric cancer (G.A.M.). The next section (7 μ m) was deparaffinized, and lightly stained with hematoxylin. Tumor tissue was precisely isolated by manual microdissection under an inverted microscope using the marked HE section for target tissue identification. Isolated tissue was digested by 50 μ l of proteinase K (Qiagen Inc, Valencia, CA) containing lysis buffer at 50°C for 16hrs. Subsequently, DNA was purified with phenol-chloroform-isoamyl alcohol (Fisher Chemicals, Fairlawn, NJ) and precipitated by ethanol. Subsequent tissue sections (4 μ m) were prepared on aminopropylethoxysilane (APES) coated slides for IHC.

Analysis of CpG island methylation status

Sodium bisulfite modification (SBM) was performed on 20 μ l of sample PEAT DNA plus 1 μ g of salmon sperm DNA as a carrier. Sample concentrations of double-stranded DNA were quantified by the PicoGreen assay (Molecular Probes, Eugene, OR) prior to bisulfite treatment and were between 10 and 100 ng/ μ l. We allowed for 90% sample loss during desalting and further clean-up steps. DNA isolation was repeated if concentrations were lower than 10 ng/ μ l. If insufficient DNA could be detected the sample was not further evaluated. SBM was carried out as previously described²⁵; sulphonation incubation time was 3hrs at 60°C. Capillary array electrophoresis analysis was used after methylation-specific PCR for analysis of CpG island methylation status as previously described. Methylation-specific and unmethylated-specific primer sets were designed around the *COX-2* transcription start site (-14bp/+110bp). The primers were dye-labeled for detection using capillary array electrophoresis (CAE). Forward and reverse sequences for the methylation-specific primer set were: 5'-TTTCGGTAGCGATTAATTGTTATAC-3' and 5'-CGAAAATAAACTTTACTATCTAAAAACGTC-3', respectively. Forward and reverse sequences for the non-methylated-specific primer set were: 5'-TTTGGTTAGTGATTAATTGTTATATGA-3' and 5'-CAAAAATAAACTTTACTATCTAAAAACATC-3', respectively. Two positive (*sssl methyltransferase* treated donor lymphocyte DNA and the RL-0380 cell line DNA), and two negative (*phi-29 DNA polymerase* amplified donor lymphocyte DNA²⁶ and the FN-0028 cell line DNA) controls were included in each assay. Relative amounts of PCR products were quantified

by CAE (CEQ 8000XL, Beckman Coulter, CA) using CEQ 8000XL software version 6.0 (Beckman Coulter), as described previously²⁷. A methylation index (MI) was calculated; $MI = [(methylated\ peak\ intensity) / (methylated\ peak\ intensity + unmethylated\ peak\ intensity)]^{28}$.

Immunohistochemistry

Tissue sections were deparaffinized and endogenous peroxidase was blocked by hydrogen peroxidase-methanol for 20min. Antigen retrieval was performed by boiling the sections in 10mM citrate buffer for 10min. Sections were incubated overnight at room temperature with a monoclonal antibody against human *COX-2* (Cayman Chemical, MI) at a dilution of 1:200 (2.5 µg/mL) in phosphate-buffered saline (pH 7.4) with 1% BSA (PBS/BSA). Sections were then incubated for 30min with biotin (1:400; DAKO, Glustrup, Denmark), washed, and incubated for 30min with Streptavidin-Biotin-Complex (SABC) (1:100; DAKO, Denmark). The sections were washed in PBS for 15min, rinsed in Tris/HCl-buffer (pH 7.6) for 5 min, and developed in 3.3 diaminobenzidine tetrahydrochloride (DAB) with hydrogen-peroxide for 10min. The sections were counterstained with hematoxylin and mounted. *COX-2* IHC staining intensity of tumor cell cytoplasm was scored independently in a blinded manner by two expert gastric cancer pathologists (GAM and NCvG) using the following scoring criteria: absent staining; weak diffuse cytoplasmic staining (may contain stronger intensity in <10% of the cancer cells); moderate granular cytoplasmic staining in 10%-90% of the cancer cells; and strong granular staining in more than 90% of the tumor cells according to the method of Buskens et al²⁹. In case of disagreement, a third independent staining assessment (by PdH) was used to designate tumor staining-intensity.

MSP assay validation

Methylation status of the *COX-2* promoter region was confirmed in gastric cancer lines, KATO-III (ATCC, Manassas, VA) and FN-0028 (JWCI), by direct bisulfite sequencing, as described previously²⁸. Forward and reverse sequencing primers were 5'-TAAGGGGAGAGAGGGAAAA-3' and 5'-CACCTATATACTAAACYCCAAAACC-3', respectively, with Y=A or G. Both cell lines were treated with 5-azacytidine (5-aza) and trichostatin-A (TSA) for verification of epigenetic regulation of *COX-2* mRNA expression, as described previously^{28,30}. *COX-2/GAPDH* mRNA expression ratio was assessed by using quantitative RealTime PCR³¹. Sequences for forward and reverse primers and fluorescent labeled probe for *COX-2* mRNA were 5'-CATTTGAAGAACTTACAGG-3', 5'-CCAAAGATGGCATCTG-3', and 5'-FAM-CTCCACAGCATCGATGTCACCATA-BHQ-3', respectively.

Study design and statistical analyses

This was a retrospective study and all assays were performed in a blinded manner to the trial clinical outcome parameters. We first established tumor-specific MI by assessment of non-neoplastic gastric tissue controls. A cut-off MI to allocate tumors to the methylated or unmethylated category was set at the 95th percentile of the measured MI values in normal controls. This cut-off was uniformly and consistently used to study the clinical value of *COX-2* methylation status initially in the pilot study and then in the validation D1D2 trial specimens. D1D2 trial patients that received resection with curative intent were selected,

satisfying the following criteria: complete surgical resection (R0) and no postoperative mortality. Required sample size of the validation patient group was calculated based on recurrence-percentages in patients with *COX-2* methylated and unmethylated tumors after 10 years of follow up of in the pilot study. Correlation between methylation status of the *COX-2* gene and clinicopathological features was analyzed by Fisher's exact test or Pearson's χ^2 test. Student's t-test evaluated differences in age between groups. The Mann-Whitney U-test was used for ordinal variables. Survival length was determined from the day of primary tumor surgery to the date of death or last clinical follow-up. The Kaplan-Meier method was used for survival analysis grouping with *COX-2* methylation status. Differences between curves were analyzed using the log-rank test. Cox's proportional hazard regression model was used in a backward stepwise method for variable selection in multivariate analyses. T-stage, N-stage, TNM-stage, trial randomization, Lauren classification, and complete resection were included in the model. Kruskal-Wallis test was used to assess the relation between *COX-2* MI and the different staining-intensity categories. The statistical package SPSS version 12.0.1 (SPSS Inc, IL) was used; a value of $P < 0.05$ (two-tailed) was considered significant.

Results

MSP assay validation

Regulation of *COX-2* expression by promoter region methylation has previously been shown in gastric cancer lines¹⁹. We first verified whether the CAE-MSP assay on *COX-2* methylation status associated with regulation of *COX-2* mRNA expression. Two representative gastric cancer lines assessed by the CAE-MSP assay as completely methylated (KATO-III, MI=1.0) and completely unmethylated (FN-0028, MI=0). The individual CpG-dinucleotide methylation status of the target region of the promoter transcription site was confirmed by bisulfite sequencing in RL-0380 and FN-0028 to be, respectively, completely methylated and unmethylated. This established these cell lines as suitable positive and negative controls for the CAE-MSP assay. RL-0380 and FN-0028 cell lines were then treated by 5-aza and TSA to evaluate *COX-2* mRNA re-expression. The combination of 5-aza and TSA was used because epigenetic silencing can involve both methylation of CpG islands and deacetylation. In KATO-III, the demethylating effect was confirmed by CAE-MSP, and expression of *COX-2* mRNA was induced (*COX-2*/*GAPDH* ratio was 0 versus 4.46E-02, respectively, before and after treatment). In FN-0028, *COX-2* mRNA was present before treatment and did not significantly change after treatment (*COX-2*/*GAPDH* ratio was 1.69E-04 versus 3.45E-04, respectively). Results confirmed that promoter region methylation affects *COX-2* expression, and validated the CAE-MSP assay for detecting methylation at the *COX-2* promoter region transcription site.

Primary tumor *COX-2* methylation status

Increased *COX-2* methylation has been shown to be a tumor-related event in gastric cancer^{32,33}. Using the *COX-2* CAE-MSP assay, we verified tumor-related *COX-2* levels. Methylation status was assessed in FAMTX patients' primary tumors, as well as 18 non-

cancerous gastric biopsies in patients with benign conditions as controls. Forty-four of the 59 patients enrolled in the trial finally underwent resection. Three of the 44 primaries could not be evaluated because of insufficient DNA, and in one patient the paraffin-embedded block had an insufficient amount of cells, leaving 40 patients available for analysis. Relatively low levels of *COX-2* methylation were detected in control samples compared to tumors (**figure 1**). The 95th percentile of the MI values in non-neoplastic gastric tissues was calculated (MI=0.24) and used as a cut-off to establish tumor-related methylation. *COX-2* methylation was detected by the CAE-MSP assay in 9 of 40 (23%) tumors using the predetermined cut-off level standard.

Primary tumor *COX-2* methylation status and *COX-2* IHC

The regulatory effect of *COX-2* promoter methylation in tumors was assessed by correlating *COX-2* gene MI values to intratumoral *COX-2* protein expression in FAMTX trial pri-

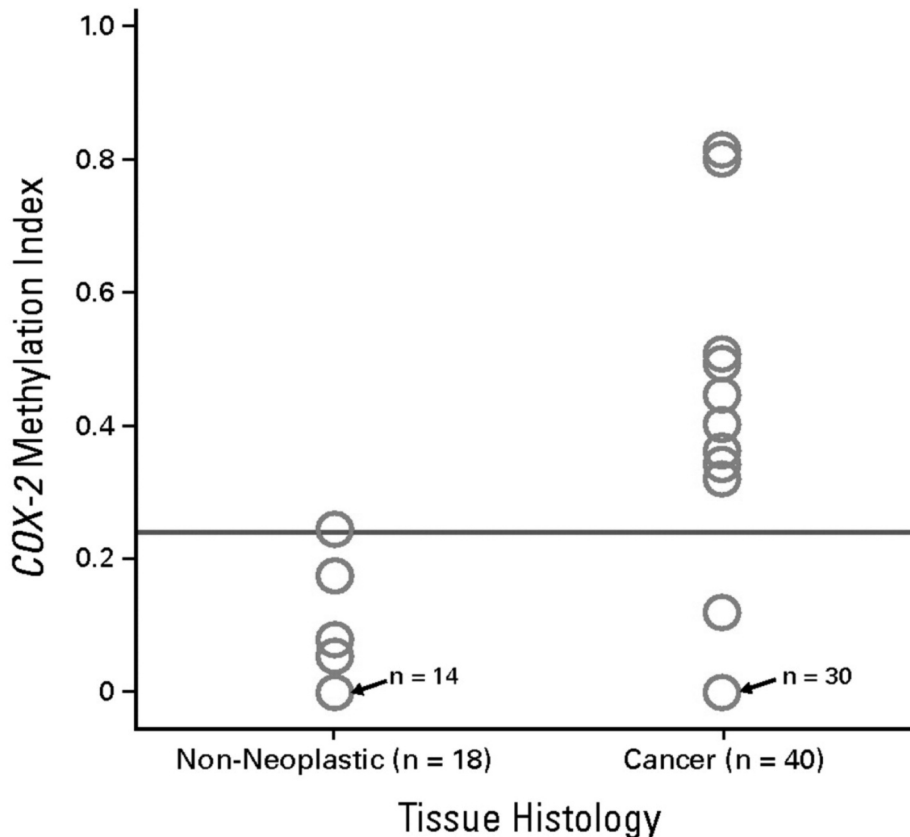


Figure 1. Scatter plot indicating distribution of measured methylation index (MI) values in normal gastric epithelium and primary gastric tumor. Horizontal bar indicates the cutoff level for increased tumor-related methylation (MI, 0.24).

maries. PEAT specimens were assessed by IHC for *COX-2* expression of tumor cells. Of the 39 evaluated cases, 3 (8%), 9 (23%), and 27 (69%) patients showed weak diffuse, moderate, and strong *COX-2* expression, respectively. **figure 2** shows representative results of *COX-2* expression with the corresponding CAE-MSP signal intensity peaks. The relation of *COX-2* gene methylation status to respective protein expression in tumor cells is shown in **figure 3**. Mean MI values were 0.46, 0.32, and 0.13 for tumors with weak diffuse, moderate granular, and strong granular staining, respectively. The gradual decrease of methylation levels along *COX-2* IHC categories increasing in staining intensity was significant ($P=0.02$ by Kruskal-Wallis test), and suggests a direct regulatory effect of *COX-2* methylation on *COX-2* protein expression in gastric tumors.

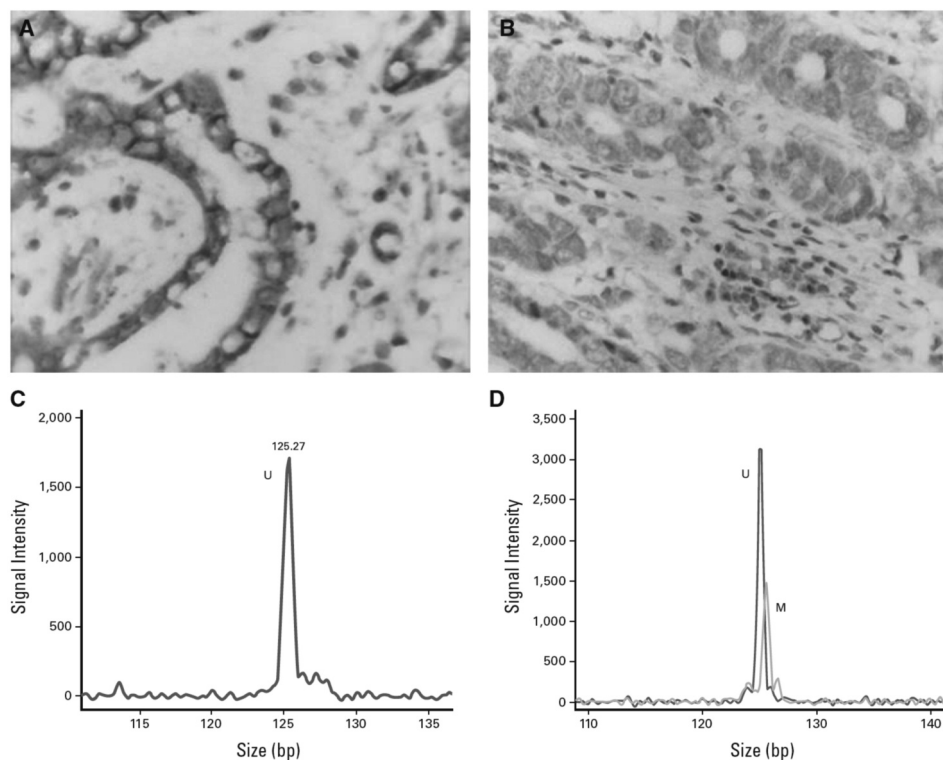


Figure 2. Representative cyclooxygenase-2 (*COX-2*) immunohistochemistry (IHC) results of primary gastric tumors with respective capillary array electrophoresis methylation-specific polymerase chain reaction (CAE-MSP) outcomes. x-axis of the CAE-MSP represents the fluorescent intensity (M, methylated product; U, nonmethylated product) indicating polymerase chain reaction amplicon. y-axis represents the product base pairs. (A, C) Nonmethylated primary gastric tumor. Strong cytoplasmic *COX-2* protein staining in tumor cells. (B, D) Methylated primary gastric tumors show weak diffuse IHC staining.

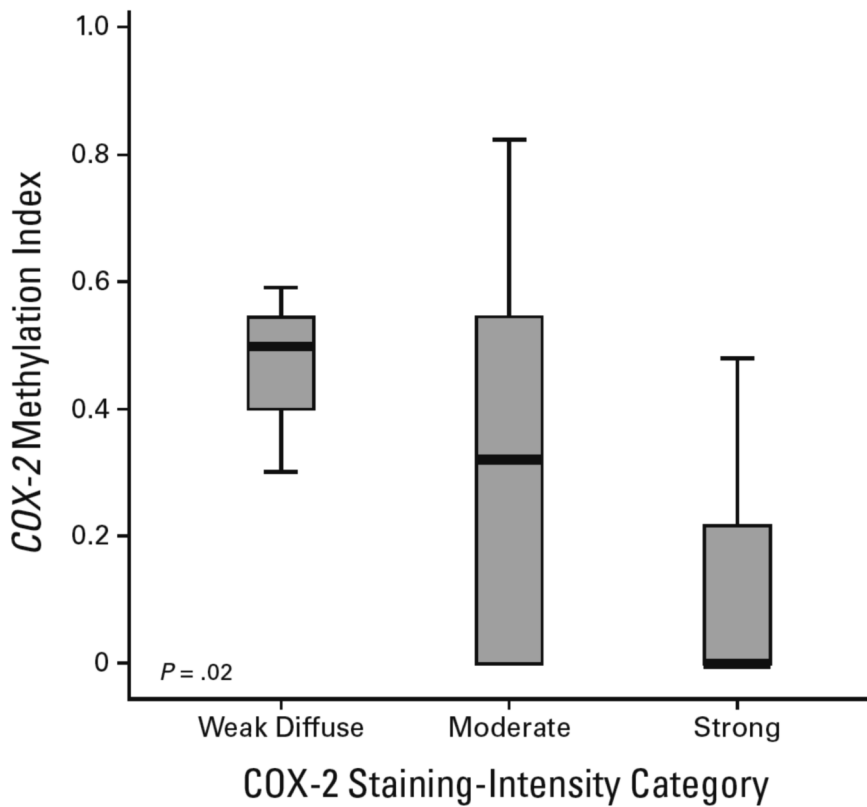


Figure 3. Boxplots showing gastric primary tumor cyclooxygenase-2 (*COX-2*) methylation index (y-axis) in relation to *COX-2* protein expression categories (x-axis) as assessed by immunohistochemistry in gastric tumor cells.

COX-2 methylation and clinical outcome

We evaluated gastric cancer patients' clinical prognostic factors between *COX-2* methylated and non-methylated FAMTX trial tumors in a pilot study. *COX-2* methylation status showed no relation to sex, age, Lauren type, T-, N- or TNM-stage, or resectability in this patient group (**table 1**). Univariate analysis of Kaplan Meier survival curves (**figure 4A-B**) demonstrated that *COX-2* methylation status gave significant differences in time to recurrence (TTR) and overall survival (OS). Five patients that did not receive a curative (R0) resection were excluded for the analysis of TTR. Multivariate analysis (**table 2**) showed that methylation of the *COX-2* gene was a favorable independent prognostic factor for TTR and OS. Nodal status showed the strongest predictive value, as expected. The multivariate analysis also showed predictive value for the FAMTX trial randomization arm, indicating improved outcome for patients that received surgery alone.

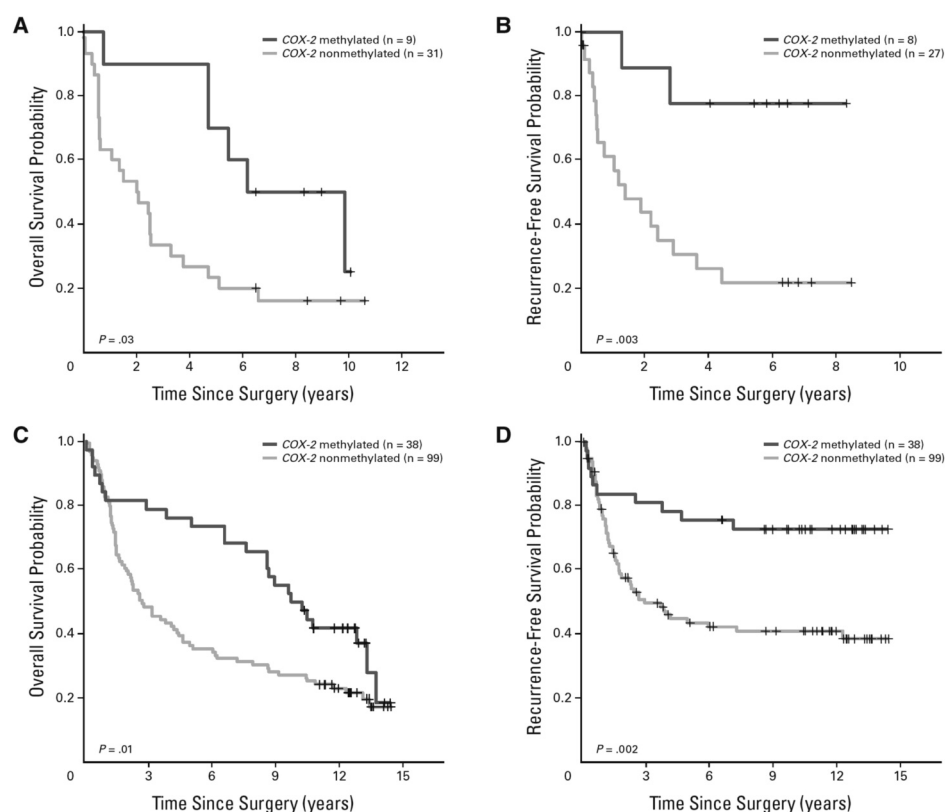


Figure 4. Kaplan-Meier analysis of survival for gastric cancer patients with primary tumors assessed for methylation status of cyclooxygenase-2 (COX-2). Among 40 fluorouracil, doxorubicin, and methotrexate (FAMTX) trial patients, those with methylated primary tumors had a significantly improved (A) overall survival and (B) longer time to recurrence for 35 patients that could be evaluated for this outcome parameter. (C, D) Represent Kaplan-Meier analysis of 137 D1D2 trial gastric cancer patients.

Based on the above pilot studies, we validated the utility of COX-2 methylation status in an independent gastric cancer population that received no neo-adjuvant treatment. To establish sample size we initially performed power calculations using the results of the test-set. Based on these values we calculated 129 patients to be sufficient to obtain significance with an alpha-level of 0.05 and 90% power. Because of expected difficulties in retrospectively analyzing (on average) 12 year old tissue blocks, we added 40% extra patients to account for patients of which no results could be generated. To obtain a homogenous study group we requested blocks of patients without postoperative mortality and that received R0 resection (resection margin microscopically free of tumor). These patients are more likely to be node-negative. An unbiased selection was performed to assure sufficient events for survival analysis. This resulted in that 37% of patients analyzed were node-negative in the

COX-2 Trial Patients						
Variable	FAMTX (n=40)			D1D2 (n=137)		
	Meth	Unmeth	P*	Meth	Unmeth	P*
No. of patients	9	31		38	99	
Sex						
Female	5	15	.84	19	47	.79
Male	4	16		19	52	
Age, years						
Mean	61.7	60.1	.58†	66.9	67.2	.85†
SD	6.6	9.9		8.7	8.2	
Randomization assignment						
FAMTX surgery/D1 resection	4	15	.84	24	54	.36
Surgery alone/D2 resection	5	16		14	45	
Lauren classification						
Intestinal	6	17	.59	33	73	.34
Diffuse	3	14		4	23	
Mixed	—	—		1	3	
UICC pathologic stage						
IA	2	2	.43‡	15	14	.008‡
IB	2	5		8	23	
II	2	15		7	30	
IIIA	3	4		5	21	
IIIB	0	1		1	6	
IV	0	4		2	5	
UICC nodal status						
N0	4	12	.71‡	23	28	.006‡
N1 (1-6 positive)	5	15		9	52	
N2 (7-15 positive)	0	3		4	16	
N3 (16 positive)	0	1		2	3	
Tumor invasion						
T1	2	2	.68‡	15	24	.02‡
T2	4	19		20	52	
T3	3	10		3	21	
T4	—	—		0	2	
Curative resection						
Yes	8	27	.89	—	—	—
No	1	4		—	—	

Table 1. Association Among COX-2 Methylation and Clinicopathologic Variables

Abbreviations: COX-2, cyclooxygenase-2; FAMTX, fluorouracil, doxorubicin, and methotrexate; Meth, methylated COX-2 promoter; Unmeth, nonmethylated COX-2 promoter; UICC, International Union Against Cancer; SD, standard deviation. Correlations by Fisher's exact test; †calculated by t test; ‡calculated by Mann-Whitney U test.

Trial	Time to Recurrence			Overall Survival		
	HR	95% CI	P	HR	95% CI	P
FAMTX						
T stage	—	—	.73	—	—	.59
No. of involved nodes			.01			.04
N0 (0)	1			1		
N1 (1-6)	3.70	1.15 to 11.96	.03	3.66	1.31 to 10.14	.01
N2 (7-15)	5.28	0.53 to 52.91	.16	3.10	0.58 to 16.62	.19
N3 (16)	159.59	6.61 to 3852.78	.002	16.18	1.42 to 184.90	.03
TNM stage	—	—	.60	—	—	.53
Curative resection	NA		NA	—	—	.39
Randomization assignment (surgery alone)	0.23	0.08 to 0.73	.01	0.30	0.12 to 0.71	.008
Intestinal type (Lauren)	—	—	.18	—	—	.09
COX-2 methylated	0.08	0.01 to 0.65	.02	0.37	0.14 to 1.00	.05
D1D2						
T stage	—	—	.16	—	—	.31
No. of involved nodes			<.0001			<.0001
N0 (0)	1			1		
N1 (1-6)	8.67	3.36 to 22.37	<.0001	1.68	1.08 to 2.67	.03
N2 (7-15)	24.25	8.66 to 67.87	<.0001	4.53	2.45 to 8.38	<.0001
N3 (16)	68.91	18.32 to 259.27	<.0001	11.54	4.17 to 31.92	<.0001
TNM stage	—	—	.17	—	—	.38
Randomization assignment (D1 resection)	—	—	.65	—	—	.85
Intestinal type (Lauren classification)	0.57	0.35 to 0.95	.03	0.63	0.42 to 0.93	.02
COX-2 methylated	0.49	0.24 to 0.99	.05	0.62	0.38 to 0.99	.05

Table 2. Multivariate Analysis of Prognostic Factors As Covariables with COX-2 Methylation Status for Gastric Cancer Disease Outcome

NOTE: Stepwise Cox regression model.

Abbreviations: COX-2, cyclooxygenase-2; HR, hazard ratio; FAMTX, fluorouracil, doxorubicin, and methotrexate; NA, not applicable.

Patients Receiving R0 Resection Without Postoperative Mortality From the D1D2 Trial (n=595)					
Factor	Nonanalyzed (n=458)		Analyzed (n=137)		P
	No.	%	No.	%	
Sex					
Female	189	43	66	48	.31
Male	260	57	71	52	
Age, years					
Median	63		66		
SE	0.6		0.7		.001
Random assignment					
D1 resection	247	54	78	57	.54
D2 resection	211	46	59	43	
T stage					
T0	5	1	0	0	.49
T1	131	28	39	28	
T2	213	47	74	53	
T3	99	22	22	17	
T4	10	2	2	2	
N stage					
N0	229	50	51	37	.05
N1 (1-6)	163	36	61	44	
N2 (7-15)	47	10	20	15	
N3 (16)	19	4	5	4	

Table 3. Comparison of Clinical Characteristics Between Selected and Non- Selected Cases From Curatively Resected Patients of the D1D2 Trial

*Calculated by Mann-Whitney U test.

validation group (similar as in the test-set). Finally, 178 blocks were processed. Molecular data could be generated of 137 patients. The patients in analysis were then assessed for differences from the non-analyzed patients (**table 3**). As expected, the selected study group showed significant higher proportion of node-positive patients. Older gastric cancer patients are more likely to be node-positive at time of surgery and this explained the higher age of the 137 analyzed patients. Thirty-eight (28%) patients were classified as *COX-2* methylated using the pilot study cut-off (MI=0.24). Mean MI was 0.71 (S.D. 0.29, range 0.26-1.00) in methylated tumors and 0.01 (S.D. 0.04, range 0-0.19) in unmethylated primaries. In this validation group, significant correlation was observed for *COX-2* methylated tumors with lower TNM-stage, node negativity, and lower T-stage (**table 2**). The predictive value of methylation status was confirmed in univariate and multivariate analysis for OS and TTR (**figure 4, table 2**).

Nodal involvement was the strongest prognostic variable and was present in all patients with methylated *COX-2* that relapsed within 1.8 years following surgery. Patients with *COX-2* methylation had improved TTR when only node-negative patients were analyzed ($P=0.03$). Lauren type, showing borderline significance in the FAMTX-trial, was selected as an independent factor in the larger study group. T-stage was not a prognostic factor and this may be due to the tight association with nodal status ($P<0.0001$). Because of this dependence, TNM-stage was not a significant factor either in multivariate analysis. Trial randomization to extended or limited nodal dissection had no predictive value as shown in the initial trial analyses²⁴.

Discussion

In gastrointestinal cancers, the role of *COX-2* in tumor-promotion has been shown^{34,35}. Numerous stimulatory factors, such as growth factors and cytokines, have been reported to cause overexpression of *COX-2* in cancer^{34,36-38}. Studies have suggested methylation as a regulatory mechanism of *COX-2* expression in gastric cancer *in vitro* and in primary tumors^{19,33,39-41}. Recently it was reported that C/EBPbeta (CCAAT/enhancer-binding protein beta) transcription factor regulates the expression of endogenous *COX-2* in a gastric cancer line model, based on its methylation status⁴². The *COX-2* methylated status may abolish the effect of factors, such as C/EBPbeta, growth factors, or cytokines, due to the inactivation of binding elements at the promoter region. Furthermore, *COX-2* methylation status in our study was independent in multivariate analyses of nodal status which is a highly important predictor of disease recurrence. This independence may be explained by some tumor-enhancing processes intrinsic to gastric adenocarcinoma that increased *COX-2* expression is reported to be associated with. Examples of such processes are angiogenesis, reduced apoptosis and increased inflammation. Also, our data suggests that *COX-2* to be involved in Lauren's histologic classification. In the pilot study, intestinal type tumors correlated to decreased IHC staining intensity ($P=0.03$), and absence of *COX-2* expression in the intestinal tumor type has been previously reported²⁰. In the D1D2 trial patient group diffuse type tumors had a significantly decreased MI compared to intestinal type tumors ($P=0.03$). Together, methylation of *COX-2* may cause gastric tumor cells to maintain a more differentiated, intestinal organization, and subsequently these tumors may constitute a clinically less aggressive tumor type.

The outcome of both trials, for which patient specimens were used in this study, was negative. The FAMTX trial did not show improved resectability rate of gastric cancer by preoperative chemotherapy, and extended D2 nodal dissection did not reduce recurrence rates, indicating the problem of managing gastric cancer disease. Development of molecular biomarkers for primary gastric cancer tumors may allow for better management strategies. Long-term analyses of the D1D2 trial suggested that node-positive gastric cancer patients may benefit from more extensive nodal dissection at primary tumor surgery²⁴, and two large randomized trials have now shown the benefit of (neo-)adjuvant treatment for gastric cancer^{3,4}. Our study confirms that presence of nodal involvement remains the single most important factor to base treatment decisions upon postoperatively. However, the D1D2 trial data showed that 20% of node negative patients show disease recurrence wit-

hin 3 years after surgery. Biomarkers to select poor-prognosis, node-negative patients that could be potentially useful for stratification in patients receiving adjuvant or neoadjuvant therapy. *COX-2* methylated tumors did show significantly less recurrence in univariate analysis in node-negative patients only, however, our study was underpowered to show independence of *COX-2* methylation status as a predictor of poor prognosis in node-negative patients. The actual advantage of our study for clinical applicability lies in that *COX-2* methylation status is a primary tumor characteristic and therefore can be assessed in standard performed diagnostic tumor biopsy. Primary tumor *COX-2* methylation status therefore may be used as a preoperatively assessable biomarker to tailor more aggressive treatment modalities at time of surgery to gastric cancer patients with poorer prognosis. To date no such biomarkers are preoperatively available in gastric cancer.

DNA is much more stable in archived tissues as compared to proteins. Our approach to assess gene *COX-2* methylation instead of protein expression by IHC analysis may be more promising as a biomarker in assessment of archival specimens. Furthermore, our study is the first in gastric cancer to show that DNA methylation is an independent prognostic factor predicting a favorable effect on patient outcome by down-regulating the expression of a known tumor-enhancing protein. Our results shift the paradigm that tumor acquired DNA methylation of promoter regions results in adverse outcome in gastric tumors.

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CHAPTER 6

Quantitative Analysis of Methylation of Genomic Loci in Early Stage Rectal Cancer Predicts Distant Recurrence

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Abstract

Introduction: There are no accurate prognostic biomarkers specific for rectal cancer. Epigenetic aberrations, in the form of DNA methylation, accumulate early during rectal tumor formation. In a preliminary study, we investigated absolute quantitative methylation changes associated with tumor progression of rectal tissue at multiple genomic methylated-in-tumor (MINT) loci sequences. We then explored in a different clinical patient group whether these epigenetic changes could be correlated with clinical outcome.

Methods: Absolute quantitative assessment of methylated alleles (AQAMA) was used to assay methylation changes at MINT1, 2, 3, 12, 17, 25, and 31 in sets of normal, adenomatous and malignant tissues from 46 patients with rectal cancer. Methylation levels of these biomarkers were then assessed in operative specimens of 251 patients who underwent total mesorectal excision (TME) without neoadjuvant radiotherapy in a multicenter clinical trial.

Results: Methylation at MINT 2, 3, and 31 increased 11-fold ($P=0.005$), 15-fold ($P<0.001$), and 2-fold ($P=0.02$), respectively, during adenomatous transformation in normal rectal epithelium. Unsupervised grouping analyses of quantitative MINT methylation data of TME trial patients demonstrated two prognostic subclasses. In multivariate analysis of node-negative patients, this subclassification was the only predictor for distant recurrence (HR:4.17, 95%CI:1.72-10.10, $P=0.002$), cancer-specific survival (HR:3.74, 95%CI:1.48-9.43, $P=0.003$) and overall survival (HR:2.68, 95%CI:1.41-5.11, $P=0.005$).

Conclusion: Methylation levels of specific MINT loci can be used as prognostic variables in patients with AJCC stage I and II rectal cancer. Quantitative epigenetic classification of rectal cancer merits evaluation as a stratification factor for adjuvant treatment in early disease.

Introduction

Rectal cancer is the second most common cancer of the digestive system in the U.S.A.¹ Neoadjuvant therapy has improved local control of rectal cancer in patients undergoing total mesorectal excision (TME)²⁻⁴, but distant recurrence remains the major cause of disease mortality. Although tumor involvement of regional nodes is the most important predictor of metastasis, 20% of node-negative patients will recur at distant sites. This suggests that even early stages of tumors have potential for systemic metastasis and, therefore, molecular subclassification may be clinically relevant. Development of prognostic molecular biomarkers for rectal cancer would improve management and potential treatment stratification. Colon and rectal cancers are often assessed together in the analysis of molecular/genetic biomarkers. This is often due to the limited availability of tumor for analysis, or specimens are not procured from a specific clinical trial. We now know both cancers are different in etiology and treatment, as well as (epi)genetics⁵. In this study, we have focused specifically on epigenetic changes of rectal cancers from a clinical trial.

Epigenetic instability, such as changes in genomic DNA methylation status, is an early event during gastrointestinal tumor development and encompasses both hyper- and hypomethylation changes⁶⁻⁸. The majority of the studies assessing epigenetic changes and association with clinical outcome focus on hypermethylation of specific genomic loci and use non-quantitative measures. Consequently, methylation status is mostly analyzed in a dichotomous manner, categorizing specimens as methylated or unmethylated. Absolute quantitative interpretation of methylation data would improve analysis of epigenetic events⁹. Recently, we developed an assay for absolute quantitative assessment of methylated alleles (AQAMA) and showed quantitative methylation events to be associated with colorectal tumor progression¹⁰. AQAMA measures the amount of methylated and unmethylated copy numbers simultaneously in a single reaction. The assay has excellent linearity in assessing DNA methylation levels and can be used on paraffin-embedded archival tissue (PEAT) sections treated with the on-slide (*in situ*) sodium bisulfite modification (SBM) technique that allows microdissected histology-oriented assessment of small (1-2mm²) lesions^{11,12}. This allows efficient comparison of precursor adenoma and normal cells adjacent to tumor cells.

Methylation levels of methylated-in-tumor (MINT) loci have not been specifically tested for prognostic utility in rectal cancer. MINT loci are CpG-dinucleotide-rich regions located in non-protein-encoding DNA regions, and have been reported to become methylated in a tumor- and adenoma-specific manner in gastric and colon cancer¹³⁻¹⁷. In a preliminary study, we quantified methylation levels of seven MINT loci at different stages of rectal tumor formation comparing paired normal-adenoma and adenoma-cancer tissues, and subsequently analyzed whether methylation level changes related to rectal tumor progression. Our developed *hypothesis* was that methylation levels at MINT loci have prognostic significance for early rectal cancer progression. We then assessed the potential prognostic utility of MINT loci in primary tumor tissues from patients enrolled in a multicenter, randomized, surgical clinical trial. In this translational study analysis, unsupervised cluster analysis identified a subclass of patients whose quantitative methylation data were independently prognostic of progression to distant disease.

Materials and Methods

Tissue Specimens

In the preliminary study, patients operated on for rectal cancer with histopathologic confirmed adenocarcinoma were identified from the cancer registry database at SJHC. Only patients operated after 1995 were evaluated because of possible DNA degradation. Further selection of specimens was based on pathology-documented presence of tumor, as well as adenoma cells on the same tissue section.

For the clinical correlation studies, primary tumor PEAT specimens were obtained from 322 non-irradiated patients enrolled in the multi-center, randomized, quality-controlled TME trial coordinated by the Dutch Colorectal Cancer Group³. The trial investigated whether neoadjuvant radiotherapy (5x5Gy) before TME improved local control compared to TME surgery alone in patients with all stages of rectal cancer. Trial eligibility criteria and follow-up protocols have been described previously^{3,18,19}. All TME trial patients enrolled at the Dutch multicenter study sites were eligible further adhering to the following criteria: non-irradiated, TNM-stage I-III, with no evidence of disease (NED) after surgery. We opted to analyze the treatment arm because potential effects of radiation on genomic methylation are not known. Research protocols for the methylation studies on PEAT were approved by SJHC/JWCI and Leiden University Medical Center IRBs.

DNA preparation from PEAT specimens for preliminary and clinical studies

From the preliminary study specimens, two consecutive sections (4 and 7 μ m) of each PEAT block were cut and placed on adhesive-coated slides. The 4 μ m section was stained with H&E and mounted. Tissue areas with normal epithelial, classic adenomatous, and invasive cancer cells were identified and marked off by an expert surgical pathologist (R.R.T). The tissue categories were histopathology identified. Cancer cells were only taken from areas with nuclear atypia and signs of invasion of tissue architectural boundaries, the hallmark of cancer. Adenomatous cells were only taken from areas with classic villous and/or tubular adenomatous dysplasia. We did not include adenomatous tissue in the analysis with highly-dysplastic features without signs of invasion. The 7 μ m section was treated by on-slide SBM as described previously¹¹. Target tissue areas were identified and microdissected under a light microscope. Isolated cells were digested and 1 l of the lysate was used for PCR.

From the clinical study TME trial patient specimens, tissue sections (7 μ m) were cut from PEAT specimens and mounted on non-adhesive glass slides. Tumor-representative areas on H&E stained sections were marked by a surgical pathologist specializing in rectal cancer (JHJMvK). Two sections per patient were deparaffinized, and the marked tissue was carefully microdissected. DNA was isolated and modified by sodium bisulfite, as previously described²⁰. Salmon sperm DNA was added as a carrier²¹. DsDNA and ssDNA were quantified before and after SBM by PicoGreen and OliGreen assays (Molecular Probes), respectively. Sufficient input DNA for AQAMA was determined as described¹⁰. A salmon sperm DNA sample without tumor DNA was included in triplicate to assess background signal. Tissue blocks and isolated DNA were coded to prevent any bias.

AQAMA MINT locus methylation level assessment

Absolute quantitative assessment of methylated alleles at MINT loci 1, 2, 12, and 31 has been described previously¹⁰. Unpublished primer and probe sets for the remaining three MINT loci were: MINT3, 5'-TGATGGTGTATGTGATTTGTGTT-3'(forward), 5'-ACCC-CACCCCTCACAAAC-3'(reverse), 5'-ACCTACGAACGAACAC-3'(methylated probe), 5'-TACCTACAAACAAACAC-3'(unmethylated probe); MINT17, 5'-AGGGGTTAGGTT-GAGGTTGTT-3' (forward), 5'-TCTACCTCTTCCCAAATTCCA-3'(reverse), 5'-TTG-GATGGATCGCGG-3' (methylated probe), 5'-TATTTTGGATGGATTGTGG-3'(unmethylated probe); MINT25, 5'-GGGGATAGGAAGATGGTTT-3'(forward), 5'-CCCCATCCCATA-CAACC-3'(reverse), 5'-TTTGTTTCGTAGCGGAGT-3'(methylated probe), 5'-GATTTT-GTTTTGTAGTGGAG-3' (unmethylated probe). DNA samples were run in 384-well microplates in triplicate, and each plate contained individual marker cDNA standards with known copy numbers, allowing assessment of absolute methylated and unmethylated copy number. Controls for specificity of AQAMA for methylated and unmethylated sequences, as well as controls for non-specific amplification, were included^{10,22}. Final analysis outcome was the methylation index (MI), calculated as: $[\text{copy number}_{\text{methylated alleles}}/(\text{copy number}_{\text{methylated alleles}} + \text{copy number}_{\text{unmethylated alleles}})]$.

Profiling by Unsupervised Random Forest Clustering

For identification of patient clusters with similar MINT locus methylation profiles, we employed unsupervised random forest (RF) clustering²³, as it has been successfully applied in comparable data sets (**supplemental appendix 1**)^{24,25}.

Results

MINT Locus Methylation Levels During Rectal Cancer Development

Sets of normal, adenomatous, and malignant PEAT tissues from 46 patients with rectal cancer were examined by AQAMA of MINT loci known to be differentially methylated in colorectal cancer¹². The H&E-stained sections cut from the tissue blocks that, according to the diagnostic pathology report, contained adenoma as well as cancer tissue, were histopathology-evaluated by an expert pathologist (R.R.T). In the 46 tissue sections, 19, 46, and 35 areas of normal epithelium, adenoma, and cancer tissue, respectively, were identified. This resulted into paired analyses of 19 normal-adenoma sets and 35 adenoma-cancer sets. **Figure 1** shows scatterplots of the MI values in the three histopathology categories for each MINT locus. MINT loci 2, 3, and 31 underwent a significant increase in absolute mean methylation level during adenomatous transformation. There were no significant MINT methylation changes for any MINT locus during progression from adenoma to cancer. Subsequently, the significant increases were early events associated with dysplastic change of normal rectal epithelium. Because three MINT loci (2, 3, 31) showed significant increase in methylation levels and the normal distribution of the quantitative methylation data sets in healthy rectal epithelium changes to non-normal in adenoma in four other loci (1, 12, 17, 25) (**supplemental appendix 2**), all seven MINT loci were considered to have potential utility to identify epigenetic subclasses in the clinical study patient group.

Sample size calculations

To establish the sample size for the clinical study, we performed power calculations using methylation results of the preliminary study and recurrence rates of the TME trial. It was calculated that 250 patients were sufficient to obtain significance for predicting distantre-

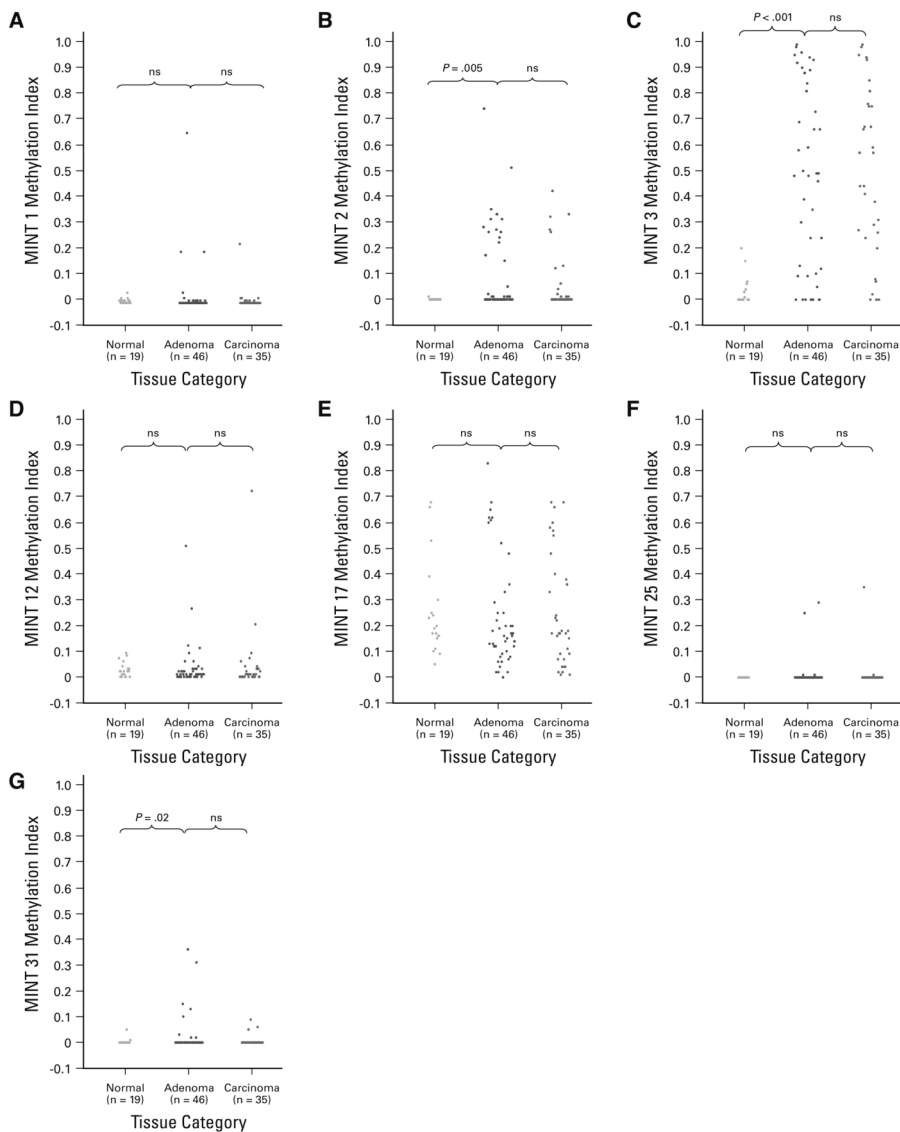


Figure 1. Scatterplots of measured methylation indices in normal rectal epithelium, rectal adenoma tissue, and rectal cancer tissue for the 7 MINT loci studied. MINT, methylated in tumor; ns, not significant.

currence with an alpha-level of 0.05 and 90% power. Because the available patient specimens from the trial were primary tumor PEAT blocks from various hospital sites, we allowed for 30% loss of cases due to availability and quality of tissue and DNA. We therefore required 72 additional cases, and the final sample size was set at 325 cases. 672 patients fulfilled our study criteria (see Material and Methods). Finally, of 314 cases, DNA was isolated (in 11 cases, tumor cell number was insufficient). Subsequently, of the 314 DNA isolations, after processing and bisulfite treatment, only 251 had sufficient input DNA for AQAMA. Characteristics of the 251 patients finally analyzed were not significantly different in prognostic factors and characteristics from the original trial population (**supplemental appendix 3**).

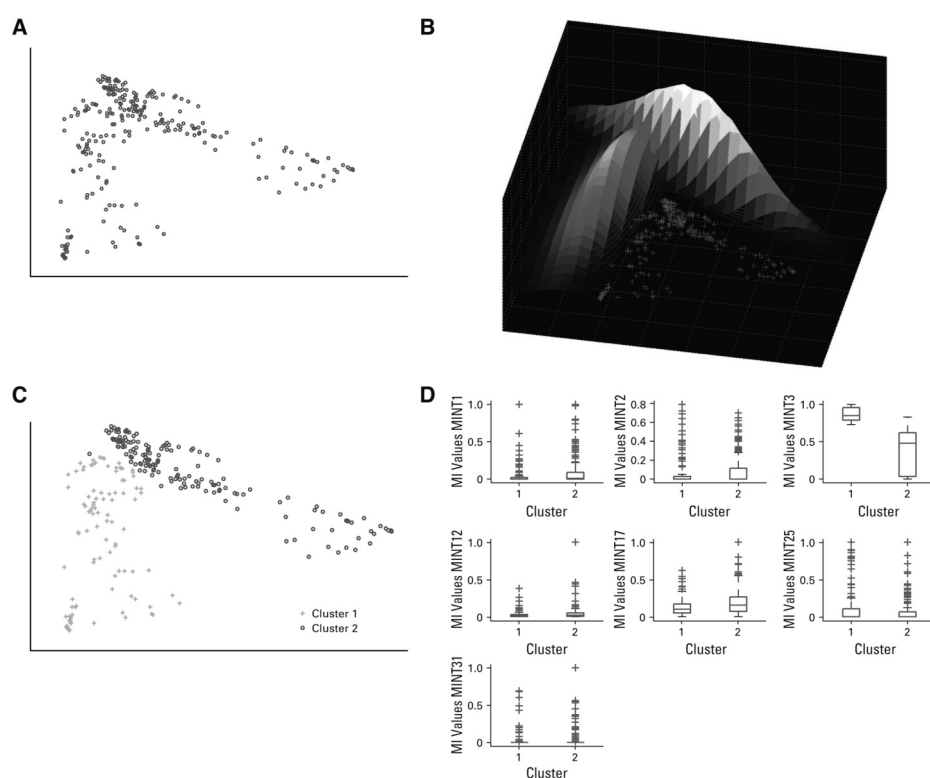


Figure 2. Methylated in tumor (MINT) locus methylation subclass identification. (A) multidimensional scaling [MDS] plot displaying the level of dissimilarity between all patients (MDS plot axes represent arbitrary units, and are therefore dimensionless). (B) Three-dimensional plot representing expectation maximization algorithm with a mixture of Gaussians analysis of the MDS plot coordinates showing Gaussian distribution (bell-shaped) of the two identified clusters. (C) MDS plot showing final cluster allocations for the patient population. (D) Box plots comparing the differences in methylation levels (MI) between cluster 1 and 2 for all MINT loci.

MINT Locus Methylation Profile Identification

To investigate whether rectal cancer can be grouped by methylation level at specific MINT loci, we performed unsupervised RF clustering on the quantitative methylation level results of patients from the TME trial. As an outcome, a MDS plot indicated the mutual distance between the cases based on methylation level of all seven MINT loci (**figure 2A**). Inspection of the MDS plot indicated two groups of rectal cancer cases. To identify which patients belonged to which group, we performed an EM-MoG analysis based on the Gaussian shape of patient clusters (**figure 2B,C**). The EM-MoG algorithm allocated the patients based on the likelihood to fall under the normal (Gaussian) distribution of one of the two clusters. Subsequently, variable importance and the methylation patterns matching the identified clusters were analyzed (**figure 2D, table 1A**). The 89 patients (35%) allocated to cluster

All Patients		Node-Negative Patients					
MINT locus	Gini Index	(n=251)		P- Value*	(n=145)		P-Value*
		Cluster 1 (n=89)	Cluster 2 (n=162)		Cluster 1 (n=55)	Cluster 2 (n=90)	
		Median†	Median†		Median†	Median†	
MINT1	11.6	0.00 (0.00-0.01)	0.01 (0.00-0.09)	<0.001	0.00 (0.00-0.02)	0.00 (0.00-0.09)	0.006
MINT2	10.8	0.08 (0.00-0.02)	0.00 (0.00-0.12)	0.07	0.00 (0.00-0.03)	0.00 (0.00-0.10)	0.51
MINT3	20.2	0.87 (0.79-0.99)	0.50 (0.06-0.65)	<0.001	0.84 (0.79-0.99)	0.49 (0.06-0.65)	<0.001
MINT12	13.5	0.03 (0.00-0.02)	0.02 (0.01-0.05)	0.01	0.02 (0.00-0.02)	0.02 (0.00-0.05)	0.22
MINT17	20.7	0.08 (0.04-0.13)	0.21 (0.08-0.30)	<0.001	0.09 (0.05-0.15)	0.20 (0.12-0.24)	0.005
MINT25	12.1	0.00 (0.00-0.04)	0.00 (0.00-0.08)	0.21	0.00 (0.00-0.05)	0.00 (0.00-0.09)	0.81
MINT31	6.0	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.90	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.82
† Interquartile range							
* Calculated by Mann-Whitney's u-test							
Table 1A: Variable Importance by Gini Index and Comparison of Mean MINT Locus MI Values Between Identified Clusters							

1 had significantly increased methylation at MINT3 and significantly decreased methylation at MINT1, 12, and 17 as compared with patients in cluster 2. The unsupervised clustering results showed that subclasses of rectal cancers can be identified by differences in DNA methylation level of tested MINT loci. The Gini-index indicated that MINT3 and MINT17 were the most important variables in forming the clusters.

Clinicopathological correlation and distant recurrence analyses

There were no significant associations observed in epigenetic subclasses of rectal cancer to any of the investigated standard clinical or tumor-pathological factors (**table 1B**). The preliminary results demonstrated that methylation level differences at the specific MINT loci develop early during tumor formation. There was no significant relation between cluster allocation and clinico-pathological factors in node-negative tumors (**table 1B**). Because identification of stage I and II patients at risk for distant metastasis is highly clinically relevant and there was no dependence of the identified patient clusters to nodal status we excluded stage III patients from distant disease recurrence analyses. We assessed the probability of distant disease recurrence, cancer-specific, and overall survival (OS). Because EM-MoG analysis is a probability-based cluster assignment algorithm, we performed multiple imputation analysis to correct for cases that have a small difference in probability to be assigned to either one of the clusters. In node-negative patients, cluster 1 patients had significant increased risk for distant recurrence ($P=0.01$), shorter cancer-specific survival ($P=0.02$), and shorter OS ($P=0.05$, **figure 3A-C**). At the time of the analyses, median duration of follow-up was 7.1 years (range 2.5–9.8 years).

Multivariate Analyses

Multivariate analyses were performed to assess whether the observed prognostic value of the clusters was independent from standard prognostic variables for the complete patient group and for node-positive and negative patients (**table 2**). T-stage, N-stage, circumferential margin status, distance of the tumor to the anal verge, and tumor differentiation were considered in a Cox's regression analysis. In node-negative patients, the quantitative MINT locus methylation profile was, of the considered variables, the only selected predictive factor for distant disease recurrence and cancer-specific survival. OS was also affected by T-stage in patients without nodal involvement. Circumferential margin involvement of the tumor and short (<5 cm) distance of the tumor from the anal verge increased the risk of distant recurrence and decreased cancer-specific survival and OS in node-positive rectal cancer patients. Possible dependence of the results on any of the 42 different study sites was evaluated in the published clinical trial report²⁶ and ruled out (data not shown) also in our analyses. The multivariate results show that the identified subclass of rectal cancers is independently predictive of distant recurrence.

MINT3 and MINT17

The Gini-index indicating variable importance in RF clustering shown in **table 1A** demonstrated MINT3 and MINT17 to hold the most information to form the two clusters compared to the other five MINT loci. We continued to assess whether methylation levels at MINT3 and MINT17 have prognostic value as a separate marker set. The quantitative

Clinical and Tumor Pathology Factors	All Patients (n=251)		P-value	Node-negative Patients (145)		P-value
	Cluster 1 n=89	Cluster 2 n=162		Cluster 1 n=55	Cluster 2 n=90	
Sex						
Male	64 (39)	98 (61)	0.08	38 (40)	56 (60)	0.40
Female	25 (28)	64 (72)		17 (33)	34 (67)	
Age						
Mean (SE)	64.8 (1.2)	62.5 (0.9)	0.15	65.4 (1.7)	63.4 (1.2)	0.33
TNM-stage						
I	29 (41)	41 (59)	0.22	29 (41)	41 (59)	0.40
II	26 (35)	49 (65)		26 (35)	49 (65)	
III	34 (32)	72 (68)		-	-	
N-status						
N ₀ (≥12 examined)	12 (35)	22 (65)	0.53	12 (35)	22 (65)	0.84
N ₀ /N _X (<12 examined)	44 (39)	68 (61)		43 (39)	68 (61)	
N ₁ (1-3 positive)	21 (34)	40 (66)		-	-	
N ₂ (≥4 positive)	12 (27)	32 (73)		-	-	
Differentiation						
Well	5 (28)	13 (72)	0.78	3 (33)	6 (67)	0.99
Moderately	66 (37)	110 (63)		44 (39)	70 (61)	
Poor	18 (32)	39 (68)		8 (36)	14 (64)	
Location distant recurrences						
Liver	11 (41)	16 (59)	0.62	4 (50)	4 (50)	0.37
Non-liver	20 (49)	21 (51)		11 (73)	4 (27)	
Resection type						
Low anterior	54 (32)	113 (68)	0.26	32 (34)	62 (66)	0.35
Abdominoperineal	33 (43)	44 (57)		21 (47)	24 (53)	
Hartmann	2 (29)	5 (71)		2 (33)	4 (67)	
Circumferential margin						
Negative	72 (35)	131 (65)	0.39	52 (39)	81 (61)	0.54
Positive	17 (35)	31 (65)		3 (25)	9 (75)	

Table 1B: Comparison of Clinical and Tumor Pathology Factors and MINT Locus Clusters

methylation data of MINT3 and MINT17 were entered into the RF algorithm and the resulting MDS plot is given in **figure 4A**. Four clearly separate clusters are formed and the corresponding methylation level differences between the clusters are plotted in **figure 4B**. Cluster 3 containing 67 patients (27%) corresponds to the previously identified high-risk cluster 1 as average MINT3 methylation index are relatively high and MINT17 methylation index is relatively low. In KM analysis, cluster 3 patients are shown to be at significantly increased risk for distant metastasis in node-negative patients compared to the other three clusters (**figure 4C**). In multivariate analysis the results showed that the high-risk cluster 3 was selected as the only independent factor among the variables analyzed predicting in node-negative patients distant recurrence probability (HR:2.84, 95%CI:1,22-6,62, $P=0.02$), cancer-specific (HR:3,29, 95%CI:1,33-8,12, $P=0.01$) and overall survival (HR: 2,21, 95%CI:1,13-4,29, $P=0.02$). It was concluded that patients at increased risk for distant metastasis can be defined as having tumors with a MINT3 methylation level > 0.72 and MINT17 methylation level < 0.14 . The analysis also demonstrated that the specific combination of increased methylation at MINT3 and decreased methylation at MINT17 is required for the prognostic information.

Discussion

Most studies of biomarkers in large bowel adenocarcinoma include colon, as well as rectum, even though rectal and colon cancers are treated differently. Moreover, right-sided and left-sided bowel adenocarcinomas have different molecular patterns; microsatellite instability and methylator phenotype are rarely seen in the rectum²⁷. Our data represents one of the largest clinical analyses of methylation biomarkers in rectal cancer specifically, and also demonstrates the first quantitative correlation between MINT methylation levels and disease progression.

The preliminary study demonstrated a progressive increase in methylation levels of specific MINT loci comparing normal and adenomatous rectal tissue. No significant change in methylation level at any MINT locus was detected comparing adenomatous and malignant rectal tissue. A correlation between methylation of MINT loci and development of adenomatous dysplasia has been reported¹⁷. Our data are unique, as we used paired normal-adenoma-cancer specimens, quantitative techniques, and analyzed rectal cancers only. The results of our clinical study identified two prognostic categories of rectal cancer based strictly on the absolute quantitative differences in methylation level. Our data show that methylation levels at multiple and two specific identified MINT loci are related to rectal tumor formation, and that they may be seen as surrogate markers of distant rectal cancer disease recurrence and disease survival. The role of non-coding regions have been of much interest in that they may be influential in gene encoding regions²⁸⁻³⁰. Especially interesting is that the chromosomal location (1p36) of the MINT3 locus, which undergoes methylation in most rectal adenomas, contains many cancer-related genes. Methylation of MINT loci 1, 2, 12, and 31 is often studied in relation to the CpG island methylator phenotype (CIMP) that forms a subclass of right colon tumors closely associated with microsatellite instability (MSI)³¹. In our study, the unsupervised clustering analyses did not identify a CIMP associated with hypermethylation in the selected MINT loci (data not shown). Interestingly, a combination of relative hyper- as well as hypomethylation was observed in the identified

Variable	All patients n=251		Node-negative n=145		Node-positive n=106	
	HR (95%CI)	P-value	HR (95%CI)	P-value	HR	P-value
Distant recurrence						
T-stage (3/4)	1.70 (0.92-3.16)	0.09	1.19 (0.49-2.93)	0.70	2.91 (1.01-8.37)	0.05
Nodal status (+)	2.47 (1.44-4.23)	0.001	-	-	-	ns
Circumferential margin (+)	1.87 (1.07-3.28)	0.03	2.40 (0.62-9.39)	0.21	1.77 (0.94-3.33)	0.08
Distance from anal verge >5 cm	0.71 (0.43-1.18)	0.19	1.50 (0.59-3.85)	0.40	0.50 (0.27-0.92)	0.03
Poor differentiation	1.39 (0.81-2.38)	0.23	1.24 (0.40-3.89)	0.71	1.59 (0.84-3.01)	0.16
MINT locus profile (cluster 1)	1.68 (1.03-2.73)	0.04	4.17 (1.72-10.10)	0.002	1.11 (0.59-2.09)	0.75
Cancer-specific survival						
T-stage (3/4)	2.12 (1.07-4.19)	0.03	1.88 (0.70-5.04)	0.21	2.85 (0.98-8.26)	0.05
Nodal status (+)	2.47 (1.41-4.35)	0.002	-	-	-	ns
Circumferential margin (+)	1.93 (1.09-3.41)	0.02	2.28 (0.59-8.81)	0.23	1.88 (0.99-3.56)	0.05
Distance from anal verge >5 cm	0.59 (0.35-0.99)	0.05	1.46 (0.53-4.03)	0.46	0.40 (0.22-0.75)	0.004
Poor differentiation	1.56 (0.91-2.70)	0.11	1.28 (0.41-4.06)	0.67	1.70 (0.88-3.29)	0.12
MINT locus profile (cluster 1)	1.47 (0.88-2.45)	0.15	3.74 (1.48-9.43)	0.005	0.99 (0.51-1.93)	0.98
Overall survival						
T-stage (3/4)	1.92 (1.14-3.23)	0.01	2.12 (1.05-4.29)	0.04	1.98 (0.87-4.50)	0.10
Nodal status (+)	1.88 (1.22-2.92)	0.004	-	ns	-	ns
Circumferential margin (+)	1.66 (1.02-2.69)	0.04	1.65 (0.60-4.53)	0.33	1.66 (0.95-2.91)	0.08
Distance from anal verge >5 cm	0.69 (0.45-1.06)	0.09	0.96 (0.49-1.90)	0.92	0.55 (0.32-0.95)	0.03
Poor differentiation	1.33 (0.84-2.09)	0.22	1.06 (0.45-2.47)	0.90	1.37 (0.77-2.44)	0.28
MINT locus profile (cluster 1)	1.48 (0.98-2.24)	0.06	2.68 (1.41-5.11)	0.003	1.00 (0.57-1.77)	1.00

Table 2: Multivariate Analysis Results of All Patients and Node-negative Patients

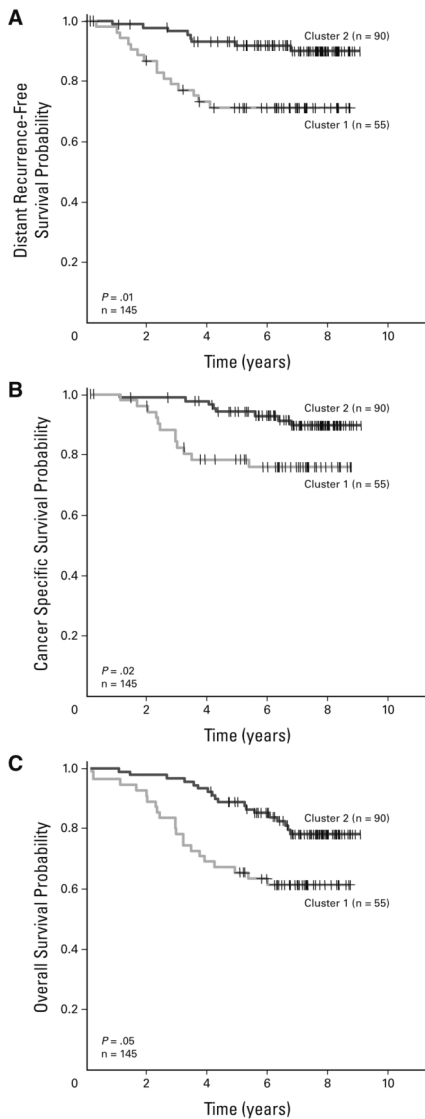


Figure 3. (A) Kaplan-Meier plots grouping analyzed node-negative total mesorectal excision trial patients into clusters 1 and 2 and comparing postoperative distant recurrence free survival probability. (B) Cancer-specific and (C) overall survival are plotted.

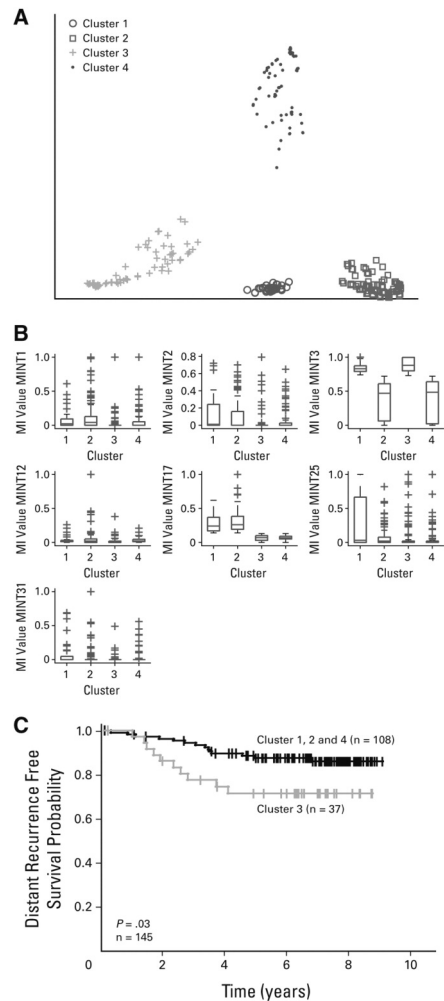


Figure 4. Random forest analyses using only MINT3 and 17 quantitative methylation data as input. (A) Multidimensional scaling plot showing the four clusters. (B) Box plots comparing the differences in methylation levels (MI) between the four clusters. (C) Kaplan-Meier plot illustrates the distant recurrence probability between high-risk cluster 3 and the combined clusters 1, 2, and 4.

subclasses. This specific combination was even required to show prognostic value on rectal cancer distant recurrence rates. This corroborates that CIMP does not occur in the rectum and that rectal cancer may have different epigenetic pathological changes compared to proximal colon adenocarcinoma. Reported correlations between MINT 1, 2, 12, and 31 and clinico-pathological features overlap with the features of MSI(+) tumors (right-sidedness, poor differentiation, early stage) and therefore our results can not be compared^{27,32,33}. We previously showed relevance of the AQAMA technique testing methylation levels at MINT 1, 2, 12, and 31 and increased methylation at this loci detected by the AQAMA assay was significantly correlated to right-sided colon tumors¹⁰.

Our preliminary study data indicates that methylation events at the measured MINT loci are related to early dysplastic proliferation of subclasses of rectal premalignancies and MINT loci may be a clinical biomarker. Subsequently, in a large rectal cancer patient group, RF clustering was able to identify, in an unbiased manner, two groups of rectal cancer patients that were naturally present within the quantitative methylation data. This demonstrated that subclassification of rectal cancer patients can be made based on absolute quantitative methylation level differences.

There was no correlation between MINT methylation profile and nodal status; in node-negative patients, the MINT profile was the only selected variable in multivariate analyses for distant recurrence probability and, subsequently, for cancer-specific survival. Identifying stage I and II patients at risk for distant disease recurrence, assessing primary tumors for predictive genomic biomarkers would be important for stratifying adjuvant treatment. Moreover, since accurate upstaging from stage II to III remains a difficult task³⁴, we approached this by a quantitative analysis of a specific panel of epigenetic biomarkers. The advantage of using genomic analysis is the stability of DNA as compared to mRNA in PEAT, where, in the latter, there is a higher level of degradation with time. Further studies will involve validation in a prospective clinical trial.

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Appendices

Supplemental Appendix 1

Profiling by Unsupervised Random Forest Clustering

Each tree is trained on a bootstrap dataset, drawn with replacement from the original dataset, and on a fixed number of randomly chosen predictors. Each tree, containing about two-third of the original dataset, places a vote on bagged data points. Bagging eliminates the need for a separate training and validation set in decision tree learning¹. The multidimensional distances are expressed in the resulting proximity matrix of the RF algorithm. A series of 5000 trees were trained with 3 random variables per node. The random forest (RF) dissimilarity algorithm used is based on individual decision tree predictors and automatically dichotomizes the expressions into clusters in a data-driven approach. The groups were therefore not established by employing cut-offs. The Gini-index indicating individual MINT loci variable importance was calculated²⁻⁴.

Cluster Assignment

We used the proximity matrix obtained from the RF clustering algorithm (represented in a multi-dimensional scaling (MDS)) plot to perform a cluster analysis.⁵ Cluster assignments were obtained by performing an expectation maximization algorithm with a mixture of Gaussians (EM-MoG) for two clusters on the scaled data. EM-MoG clustering is based on a two-step approach to fit Gaussian probability models on the data, in order to estimate most likely clusters. All analyses to identify the MINT locus profiles were performed using MATLAB software (v7.3, MathWorks) and “R” (<http://cran.r-project.org>). The number of clusters was established by inspection of the MDS-plot^{6,7}. The posterior cluster probabilities are subsequently used in a multiple imputation procedure to account for cluster membership uncertainty.

Further Statistical Analyses

In the preliminary study, differences between methylation levels of the normal, adenomatous, and cancerous tissue from the same PEAT block were assessed by non-parametric, Wilcoxon’s rank sum-test for paired samples.

For the clinical study on TME trial patients, differences in survival, clinical and tumor-pathological factors between patients assigned by RF clustering were analyzed. Specimens that did not yield sufficient DNA quantity or quality for PCR were excluded. Chi-square tests were used to compare proportions. Mann-Whitney or Kruskal-Wallis u-tests were used to compare ordinal variables. Student’s t-test was used to assess differences in age. Survival differences between groups were visualized by the Kaplan–Meier method and log-rank test assessed significance. The Cox proportional hazards model was used for multivariate analysis of time-to-event endpoints. Results are presented as hazard ratios and 95% confidence intervals. Co-variables entered in the model included T-stage, N-stage, circumferential margin status, distance of the tumor from the anal verge, and tumor differentiation. A two-sided *P*-value of 0.05 or less indicated statistical significance. All clinical correlative analyses with identified clusters were performed using SPSS statistical software (v12.0.1, SPSS

Inc, Chicago). The day 0 point for the analyses of survival and recurrence was the day of surgery. Data on patients who were alive or free of recurrence were censored at the time of the last follow-up. Uncertainty in RF cluster membership assignment was taken into account using multiple imputation⁸, where multiple ($M=5$) complete datasets were generated using the posterior cluster membership probabilities obtained from the EM-MoG algorithm.

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Supplemental Appendix 2

The distribution of the methylation data for each MINT locus for each tissue category was characterized by Kurtosis and Kolmogorov-Smirnov (K-S) analysis testing adherence to the normal distribution assumption. We have previously used this approach in the context of quantitative methylation data¹. Kurtosis indicates the size of a distribution's tail. The higher the Kurtosis, the more likely it is that the outlier MI values differ substantially from values around the mean (Kurtosis > 5 is considered significant for a sample size $n < 50$)². K-S analysis was used to test coherence of the data-set to the normal distribution assumption^{3, 4}. Significance indicates that the tested data-set is not normally distributed and values higher than the "most extreme positive difference" do significantly differ from the mean. **table 1** shows the distribution parameters of the measured methylation indices for each MINT locus and for each tissue category. MINT1, 12, and 25 methylation data distribution demonstrates high Kurtosis in adenomatous tissue while Kurtosis is lower than five in normal epithelium. K-S test becomes significant during adenomatous transformation for MINT1, 12, 17 and 25¹⁻⁴.

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			Tissue		
			Normal n=21	Adenoma n=45	Cancer n=37
MINT1	Kurtosis		1.6	31.4	34.8
	K-S analysis	Mean	0.01	0.02	0.01
		Most extreme positive difference	0.21	0.45	0.35
		P-Value	0.31	<0.001	<0.001
MINT2	Kurtosis		20.9	3.9	3.4
	K-S analysis	Mean	0.01	0.10	0.05
		Most extreme positive difference	0.49	0.35	0.38
		P-Value	<0.001	<0.001	<0.001
MINT3	Kurtosis		1.3	1.4	1.2
	K-S analysis	Mean	0.04	0.49	0.53
		Most extreme positive difference	0.31	0.14	0.09
		P-Value	0.03	0.36	0.92
MINT12	Kurtosis		0.7	21.8	25.7
	K-S analysis	Mean	0.03	0.04	0.04
		Most extreme positive difference	0.21	0.33	0.35
		P-Value	0.26	<0.001	<0.001
MINT17	Kurtosis		1.7	0.5	0.6
	K-S analysis	Mean	0.25	0.24	0.25
		Most extreme positive difference	0.26	0.24	0.19
		P-Value	0.12	0.01	0.04
MINT25	Kurtosis		n.a.	20.4	36.9
	K-S analysis	Mean	0.00	0.01	0.01
		Most extreme positive difference	0.00	0.48	0.50
		P-Value	n.a.	<0.001	<0.001
MINT31	Kurtosis		19.6	14.1	12.6
	K-S analysis	Mean	0.00	0.02	0.00
		Most extreme positive difference	0.53	0.45	0.40
		P-Value	<0.001	<0.001	0.002

*Kurtosis, Kolmogorov-Smirnov (K-S) Analysis

Table 1: Distribution results* of methylation index (MI) values in normal, adenomatous and rectal cancer tissue Marker

Supplemental Appendix 3

Characteristic	Cases in TME trial (n=1805)	Cases eligible for current study (n=672)	Selected cases for current study (n=251)	Non-selected cases for current study (n=421)
Age Category*				
Median	65	64	63	64
Range	23-92	23-92	27-85	23-92
Sex*				
Male	1151 (64)	419 (62)	162 (65)	257 (61)
Female	654 (36)	253 (38)	89 (35)	164 (39)
Type of Resection*				
None	45 (2)	-	-	-
Low anterior	1183 (66)	442 (66)	167 (66)	275 (65)
Abdominoperineal	485 (27)	206 (31)	77 (31)	129 (31)
Hartmann	90 (5)	24 (3)	7 (3)	17 (4)
Unknown	2 (<1)	-	-	-
TNM stage*				
0	28 (2)	11 (2)	-	11 (3)
I	509 (28)	203 (30)	70 (28)	133 (32)
II	497 (28)	186 (28)	75 (30)	111 (26)
III	624 (35)	272 (40)	106 (42)	166 (39)
IV	122 (7)	-	-	-
Unknown or no resection	25 (1)	-	-	-
Circumferential Margin*				
Negative	1494 (83)	552 (82)	207 (82)	345 (82)
Positive	301 (17)	120 (18)	44 (18)	76 (18)
Unknown	10 (<1)	-	-	-

* P-value not significant; patients in current study were not significantly different from the original trial population.

Table 1: Comparison of Patient Characteristics of the TME Trial and Selected Cases

CHAPTER 7

Identification of a Quantitative MINT Locus Methylation Profile Predicting Local Regional Recurrence of Rectal Cancer

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Abstract

Purpose: Risk assessment for loco-regional disease recurrence would be highly valuable in preoperative treatment planning for patients undergoing primary rectal tumor resection. Epigenetic aberrations such as DNA methylation have been shown to be significant prognostic biomarkers of disease outcome. In this study, we evaluated the significance of a quantitative epigenetic multimarker panel analysis of primary tumors to predict local recurrence in rectal cancer patients from a retrospective multicenter clinical trial.

Experimental Design: Primary tumors were studied from patients enrolled in the trial that underwent total mesorectal excision (TME) for rectal cancer (n=325). Methylation levels of seven methylated-in-tumor (MINT) loci were assessed by absolute quantitative assessment of methylated alleles (AQAMA). Unsupervised random forest clustering of quantitative MINT methylation data was used to show subclassification into groups with matching methylation profiles.

Results: Variable importance parameters (Gini-Index, GI) of the clustering algorithm indicated MINT 3 and 17 (GI=20.2 and 20.7, respectively) to be informative for patient grouping compared to the other MINT loci (highest GI 12.2). When using this two-biomarker panel, four different patient clusters were identified. One cluster containing 73% (184/251) of the patients was at significantly increased risk of local recurrence (hazard ratio 10.23, 95% CI 1.38-75.91) in multivariate analysis, corrected for standard prognostic factors of rectal cancer. This group showed a significant higher local recurrence probability than patients receiving preoperative radiation ($P<0.0001$).

Conclusion: Quantitative epigenetic subclassification of rectal cancers has clinical utility in distinguishing tumors with increased risk for local recurrence and may help tailor treatment regimens for loco-regional control.

Introduction

Rectal cancer is the second most common cancer of the digestive system in the USA¹. Worldwide, colorectal cancer has the second highest incidence², with rectal tumors constituting 33% of large bowel tumors³. An important clinical feature of rectal cancer is its close anatomical relation to the small pelvis which makes it prone to local recurrence after surgical removal even at early stage disease. Loco-regional recurrence occurs in approximately 10% of rectal cancer patients after total mesorectal excision (TME) surgery with curative intent^{4, 5}. However, loco-regional recurrence is difficult to treat with a very poor overall survival prognosis.

Fixation in the small pelvis makes malignancies of the rectum suitable for external beam radiation therapy. The Dutch multicenter TME clinical trial demonstrated significant reduction of local recurrence by adding short course (5 x 5Gy) preoperative radiation therapy to TME⁶. The multidisciplinary treatment of rectal cancer is a subject of many clinical trials randomizing patients to regimens that include various neoadjuvant therapies combined with radical surgery⁷⁻¹⁰.

(Neo)adjuvant regimens aim at two clinical outcome parameters: improvement of local control and/or to reduce distant recurrence, the latter occurring in approximately 25% of patients after radical primary tumor resection⁴. The improved local control shown by the TME clinical trial has not translated into an overall survival benefit in the trial analyses after 6 years of follow-up and the data show that overall survival is determined predominantly by distant recurrence⁴. The trial data further show that only 25% of non-irradiated tumors with distant spread also recur locally, whereas 60% of the locally recurrent tumors show distant disease spread^{4, 6}. The distant-spreading, non-locally recurring tumor may therefore constitute a separate subclass of rectal cancer.

Allocation of patients to neoadjuvant therapies might lead to overtreatment; since 10% of rectal cancer patients will develop local recurrence, 90% of patients may be overtreated. Neoadjuvant therapies used in the treatment of rectal cancer have their specific morbidities, as has been shown for both radiotherapy¹¹ and combined chemoradiotherapy¹². It is therefore of great importance to define biomarkers that can categorize tumors into local and distant spreading type preoperatively to target the multimodality treatment regimens towards a more systemic or local approach.

Molecular analysis of primary tumor tissues is an attractive form of preoperative diagnostics since rectal primary tumors are easily accessible for biopsy, which is routinely performed in the preoperative work-up. In contrast to colorectal cancer, to date few biomarkers have been described to be predictive or prognostic value specifically in rectal cancer. It is important especially in multivariate analyses to distinguish rectal cancer from other bowel adenocarcinomas or colon cancer. Specific factors need to be taken into account for rectal cancer as compared to colon cancer, such as circumferential margin involvement, type of surgical procedure or distance of the tumor to the anal verge¹³.

Epigenetic changes, such as changes in DNA methylation status, are regarded as early events contributing to carcinogenesis¹⁴. Methylation of cytosine residues in DNA is one of the mechanisms regulating transcriptional activity¹⁵. In cancer, aberrant DNA hypermethylation of specific regions as well as global hypomethylation is observed¹⁶. In this study, we

investigated epigenetic changes in rectal cancer and specifically methylation of methylated-in-tumor (MINT) loci. MINT loci are CpG dinucleotide-rich regions located in nonprotein-encoding DNA regions and have been reported to become methylated in a tumor-specific manner in (colo)rectal cancer^{17, 18}, gastric cancer¹⁹ and recently malignant melanoma²⁰.

In a previous study, we have quantitatively studied methylation of MINT loci in pre-malignant stages of rectal cancer and have shown that the MINT methylation index increases during adenomatous transformation of normal epithelium¹⁸. We also identified a quantitative, two MINT methylation biomarker panel (MINT3 and MINT17) that is predictive of distant recurrence in early, node-negative rectal cancers patients¹⁸. In the present study, we assessed the value of this panel to predict local recurrence of patients enrolled in the multicenter, randomized, clinical TME trial.

Materials and Methods

Tissue specimens

Primary paraffin-embedded archival tissue (PEAT) specimens were obtained from 325 non-irradiated patients enrolled in the TME multicenter clinical trial⁴. Patients used in this study fulfilled the following criteria: non-irradiated, TNM stage I-III, with no evidence of disease after surgical resection. Using power calculations, a sample size of 250 patients was calculated to be sufficient to obtain statistical significance for predicting recurrence (with $\alpha = 0.05$ and a power of 90%), as described previously¹⁸. Allowing for 30% loss of patient samples due to availability and quality of PEAT and DNA, 75 additional PEAT specimens were collected. In eleven patient PEAT blocks, tumor tissue was no longer present on the section. DNA was isolated from 314 randomly selected patient specimens with sufficient tumor cell numbers. After processing and sodium bisulfite treatment, samples of 251 patients yielded sufficient amount of quality DNA for AQAMA. The selected group of patients analyzed in our previous study¹⁸ did not differ from non-selected patients in the non-irradiated treatment arm or from the complete trial population. Trial eligibility criteria and follow-up protocols have been described previously^{4, 21, 22}. Non-irradiated patients were selected since predictive value of the tested biomarkers for local recurrence probability should be tested in patients who did not receive adjuvant therapy, as this affects local recurrence.

For external validation studies, 43 additional TME trial patients that adhered to the same selection criteria were added. Further, 42 non-irradiated patients that participated in the transanal endoscopic microsurgery (TEM)²³ were added and these have been previously described²⁴. DNA was extracted from adenomatous and cancer tissues. The adenomas were subdivided into cases consisting of only adenoma tissue in the resection (A, n=21) and adenoma fractions of cases with a carcinoma focus infiltrating at least in the submucosa (A, C+, n=8). The carcinomas were subdivided into groups: tumor fractions consisting of a mixture of adenoma and carcinoma tissue (C+A, n=6) and only carcinomas (C, n=7). We further collected normal rectal epithelial tissue from the tumor specimen resection margins as normal controls from 19 patients operated for rectal cancer at the Saint John's Health Center.

DNA preparation from PEAT specimens

From the TME trial patient's primary tumor PEAT specimens, 7- μ m tissue sections were cut and mounted on non-adhesive glass slides. Tumor-representative areas on H&E-stained sections were identified and marked by a surgical pathologist specializing in rectal cancer (JHJMvK). Per patient, two tissues sections were deparaffinized. DNA was isolated from microdissected tissue from the marked areas and modified by sodium bisulfite, as previously described²⁵. Salmon sperm DNA was added as a carrier²⁶. Before and after sodium bisulfite modification, double-stranded and single-stranded DNA were quantified using PicoGreen and OliGreen assays (Molecular Probes; Invitrogen, Carlsbad, CA), respectively. Sufficient input DNA for AQAMA was determined as described¹⁷. To assess background signal, a salmon sperm DNA sample without tumor DNA was included in all assays in triplicate. To prevent any bias, PEAT blocks and isolated DNA samples were coded.

AQAMA MINT locus methylation level assessment

Primary tumor methylation levels of methylated-in-tumor (MINT) loci 1,2,3,12,17,25 and 31 were assessed by AQAMA in triplicate¹⁷. Controls for specificity of AQAMA for both methylated and unmethylated sequences, as well as controls for nonspecific amplification, were included^{17, 27}. As a final outcome of the analysis, a sample's methylation index (MI) was the mean value of three MI measurements that were calculated for each well as follows: $[\text{copy number}_{\text{methylated alleles}} / (\text{copy number}_{\text{methylated alleles}} + \text{copy number}_{\text{unmethylated alleles}})]$. Of these 3 measurements the standard deviation (SD) was calculated. Values with a SD <0.1 were accepted and used in analysis. When the SD was larger than 0.1 this was in most cases due to that 1 or 2 out of the 3 methylated or unmethylated measurements was undetermined. These cases were identified and the MI was then calculated as mean methylated copy number / (mean methylated copy number + mean unmethylated copy number). If 1 out of 3 methylated or unmethylated measurements could not be determined this value was not incorporated in the calculation. If 2 or more out of 3 measurements could not be determined the value was zero. Cases with 6 complete results and with an SD > 0.1 were individually evaluated. When after omitting the most deviate MI value the SD was still > 0.1 the sample was excluded from analysis.

Statistical analysis

Differences in recurrence probability, survival and clinical and tumor pathologic factors were analyzed between TME trial patients assigned by unsupervised random forest (RF) clustering using MINT3 and MINT17 as described¹⁸. Unsupervised RF clustering dissimilarity algorithms are based on individual-decision tree predictors and it automatically dichotomizes the expressions into clusters in a data-driven approach²⁸. Groups are therefore not established by employing cutoffs. The internal validation of RF clustering further eliminates the need for separate training and validation sets to test reproducibility of cluster formation. External validation of the data in an independent patient group is still required. The GI^{29, 30} of each input variable (MINT locus) was given. This index measures the inequality of two distributions and is defined as the ratio between the area spanned by the observed cumulative distribution and the area of a uniform hypothetical cumulative distribution for a non-discriminating variable. A higher GI shows increasing inequality and therefore

higher discrimination of that input variable.

Specimens yielding insufficient or low quality DNA for polymerase chain reaction were excluded. To compare ordinal variables, Mann-Whitney and Kruskal-Wallis U tests were performed. Differences in age were assessed using t tests. Cumulative incidences, accounting for death as a competing risk, were used to visualize survival differences³¹ and significance was assessed by the log-rank test. For multivariate analysis the Cox proportional hazards model was used, with results presented as hazard ratios and 95% confidence intervals. Co-variables entered in the model included T stage, N stage, circumferential margin status, distance of the tumor from the anal verge and tumor differentiation. All clinical correlative analyses with identified clusters were performed using SPSS statistical software (version 16.0.1, SPSS Inc, Chicago, IL). A two-sided P value of 0.05 or less was considered statistically significant. Data on patients alive or free of recurrence were censored at the time of the last follow-up.

Results

MINT locus methylation profiling

In our previous study, methylation levels at the seven MINT loci were measured in normal, adenomatous and cancer rectal tissue¹⁸. The results showed that in normal tissue, all

	Cluster				Clusters	P-Value
	1 (n=33) Mean MI (95% CI)	2 (n=83) Mean MI (95% CI)	3 (n=67) Mean MI (95% CI)	4 (n=68) Mean MI (95% CI)	1,2 and 4 (n=184) Mean MI (95% CI)	
						Cluster 3 vs. Clusters 1,2 and 4
MINT1	0.09 (0.04-0.15)	0.14 (0.09-0.19)	0.04 (0.00-0.07)	0.07 (0.03-0.11)	0.11 (0.07-0.13)	<0.001
MINT2	0.14 (0.06-0.22)	0.12 (0.07-0.16)	0.05 (0.02-0.09)	0.07 (0.03-0.10)	0.10 (0.08-0.13)	0.001
MINT3	0.85 (0.82-0.87)	0.37 (0.32-0.43)	0.88 (0.86-0.90)	0.38 (0.31-0.45)	0.46 (0.41-0.50)	<0.001
MINT12	0.04 (0.02-0.06)	0.07 (0.03-0.10)	0.03 (0.01-0.04)	0.03 (0.02-0.04)	0.05 (0.03-0.06)	0.07
MINT17	0.28 (0.24-0.33)	0.31 (0.28-0.35)	0.06 (0.05-0.07)	0.07 (0.06-0.07)	0.22 (0.19-0.24)	<0.001
MINT25	0.24 (0.12-0.37)	0.08 (0.05-0.12)	0.10 (0.04-0.16)	0.07 (0.03-0.11)	0.11 (0.08-0.14)	0.04
MINT31	0.09 (0.02-0.17)	0.05 (0.02-0.08)	0.02 (0.00-0.03)	0.04 (0.01-0.07)	0.05 (0.03-0.08)	0.10

MI: methylation index. CI: confidence interval.

Supplemental Table 1: Methylation Index Values for Individual Cluster and Combined

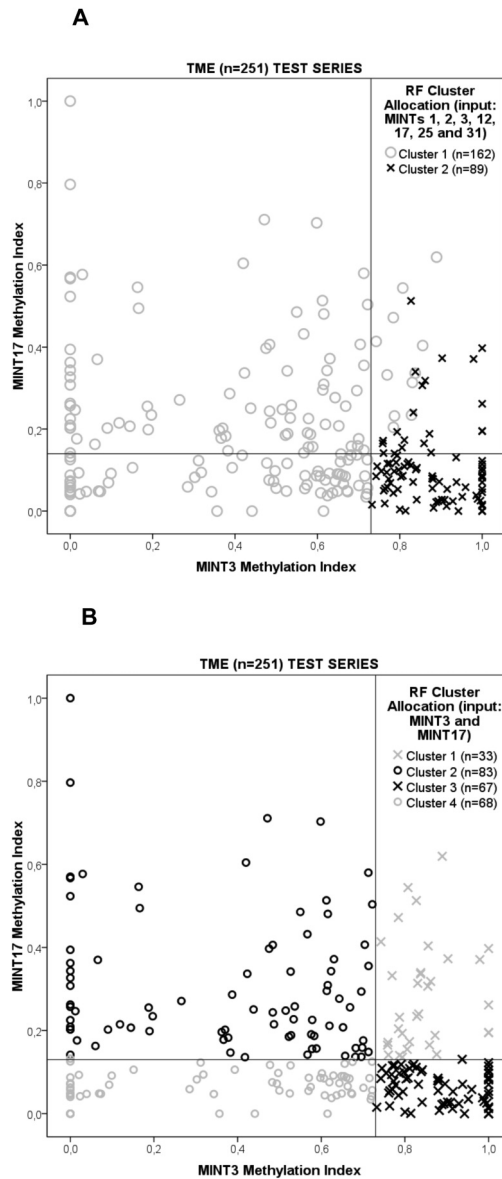


Figure 1. Methylation index plots of MINT3 and MINT17 for individual patients. Representation of MINT3 (X-axis) and MINT17 (Y-axis) methylation indices for TME trial patients using all seven and using only MINT3 and 17 in the random forest algorithm in Figures 1a and 1b, respectively. The horizontal and vertical reference lines represent cut-offs to separate the groups.

	M3 & M17			M3 & M17		
	Node Negative (n=145)		P- value	Node Positive (n=106)		P- value
Clinical parameters	Cluster 1,2 & 4 n=108	Cluster 3 n=37		Clusters 1,2 & 4 n=76	Cluster 3 n=30	
Sex						
Male	70 (75)	24 (25)	1	46 (68)	22 (32)	0.22
Female	38 (75)	13 (25)		30 (79)	8 (21)	
Age						
Mean (SE)	63.9 (1.1)	64.9 (2.0)	0.67	61.0 (1.4)	65.2 (1.9)	0.10
TNM-stage						
I	51 (73)	19 (27)	0.66	-	-	-
II	57 (76)	18 (24)		-	-	
III	-	-		76 (72)	30 (28)	
N-status						
N ₀ (≥12 examined)	27 (79)	7 (21)	0.51	-	-	0.95
N ₀ /N _X (<12 examined)	81 (73)	30 (27)		-	-	
N ₁ (1-3 positive)	-	-		44 (71)	18 (29)	
N ₂ (≥4 positive)	-	-		32 (73)	12 (27)	
Differentiation						
Well	7 (78)	2 (22)	0.88	7 (78)	2 (22)	0.91
Moderately	84 (74)	30 (26)		44 (71)	18 (29)	
Poor	17 (77)	5 (23)		25 (71)	10 (29)	
Location distant recurrences						
Liver	6 (75)	2 (25)	0.20	13 (68)	6 (32)	0.83
Non-liver	7 (47)	8 (53)		17 (65)	9 (35)	
Resection type						
Low anterior	72 (77)	22 (23)	0.55	53 (73)	20 (27)	0.76
Abdominoperineal	31 (69)	14 (31)		22 (69)	10 (31)	
Hartmann	5 (83)	1 (17)		1 (100)	0 (0)	
Circumferential margin						
Negative	98 (74)	35 (26)	0.73	54 (73)	20 (27)	0.66
Positive	10 (83)	2 (17)		22 (69)	10 (31)	
Supplemental Table 2:	Comparison of clinical and tumor pathology factors between two MINT loci clusters in node-negative and node-positive patients.					

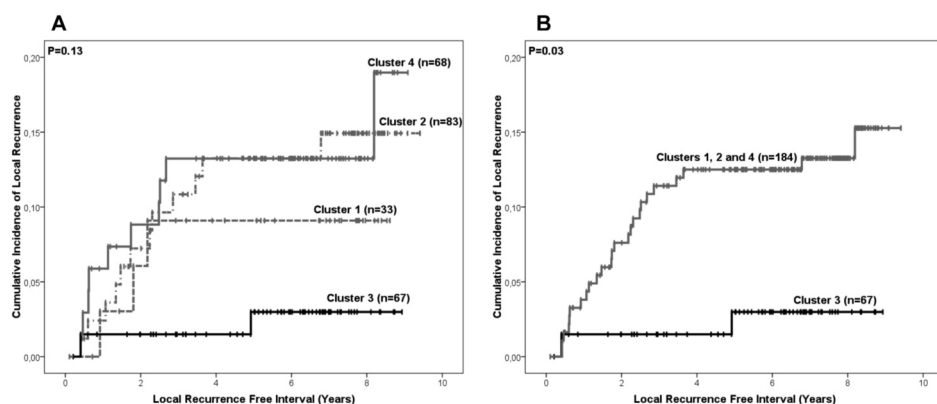


Figure 2. Cumulative incidence plots displaying local recurrence incidence for the four allocated clusters. Cumulative incidence plots showing differences in local recurrence rates between the four separate clusters in **A** and distant in **B** for cluster 3 patients compared to patients in the combined clusters 1, 2 and 4 in **C** and **D**.

MINT loci, except for MINT17, were mostly unmethylated. Significantly higher methylation of MINT2, 3 and 31 was detected in adenoma and cancer tissue compared to normal tissue. Further analysis of the quantitative data showed non-parametric distributions indicating presence of subgroups for MINT1, 2, 12, 17, 25 and 31 in adenoma and cancer tissue. Based on these findings, we concluded that all seven MINT loci had potential to subclassify rectal cancer patient groups with corresponding methylation level patterns. Quantitative methylation data of the seven MINT loci were then used to perform unsupervised RF clustering analysis. An important aspect of RF clustering is that it eliminates a validation-training approach because of its internal validation quality. External validation of the data is still required. The multidimensional scaling (MDS) plot as an outcome of using all seven MINT loci in the RF clustering algorithm suggested presence of two clusters¹⁸. The GI of the RF-analysis, indicating variable importance, appointed MINT3 and MINT17 as the two MINT loci that carried the most information to form the clusters¹⁸. Being a measure of inequality, the higher the GI, the more the clusters can be considered different based on that specific biomarker. MINT3 and MINT17 were shown to have the highest GI (20.2 and 20.7, respectively), compared to the five other MINT loci (range 6.0 - 13.5)¹⁸. The MDS plot of the RF clustering using only these two biomarkers showed four clearly separate groups¹⁸. Methylation level differences at the seven studied MINT loci between the four clusters are given in **supplemental table 1**. For a more simplified representation, individual patient MI values of MINT3 and were rendered in XY-plots. In **figure 1A** the patients are labeled according to the outcome of the RF analysis using seven MINT loci that showed presence of two clusters. In **figure 1b** the patients are labeled according to the outcome of the RF analysis using only MINT3 and MINT17 that showed presence of four clusters. **figure 1B** further shows that the groups can be divided by a cut-off of MI=0.73 for MINT3 and MI=0.14 for MINT17 with almost no miss-classification. This is important

		M3 & M17		P-value
		Cluster 1,2 & 4 n=184	Cluster 3 n=67	
Sex				
	Male	116 (72)	46 (28)	0.41
	Female	68 (76)	21 (24)	
Age				
	Mean (SE)	62.7 (0.9)	65.0 (1.4)	0.17
TNM-stage				
	I	51 (73)	19 (27)	0.78
	II	57 (76)	18 (24)	
	III	76 (72)	30 (28)	
N-status				
	N ₀ (≥12 examined)	27 (79)	7 (21)	0.67
	N ₀ /N _x (<12 examined)	82 (73)	30 (27)	
	N ₁ (1-3 positive)	44 (72)	17 (28)	
	N ₂ (≥4 positive)	32 (73)	12 (27)	
Differentiation				
	Well	14 (78)	4 (22)	0.90
	Moderately	128 (73)	48 (27)	
	Poor	42 (74)	15 (26)	
Location distant recurrences				
	Liver	19 (70)	8 (30)	0.44
	Non-liver	24 (59)	17 (41)	
Resection type				
	Low anterior	125 (75)	42 (25)	0.46
	Abdominoperineal	53 (69)	24 (31)	
	Hartmann	6 (86)	1 (14)	
Circumferential margin				
	Negative	152 (73)	55 (27)	1
	Positive	32 (73)	12 (27)	

Table 1: Comparison of clinical and tumor pathology factors between identified two MINT locus clusters in all patients

Variable	Local Recurrence	
	HR (95%CI)	P Value
T-Stage(3-4)	1.16 (0.44-3.08)	0.76
Node (+)	3.40 (1.39-8.33)	0.007
Circumferential Margin (+)	2.27 (0.93-5.53)	0.07
Distance from Anal Verge <5cm	1.40 (0.62-3.18)	0.42
Poor Differentiation	0.98 (0.40-2.41)	0.96
MINT Locus Profile*	10.23 (1.38-75.91)	0.02

HR: hazard ratio, CI: confidence interval. *: "Cluster 3" is null-hypothesis

Table 2: Multivariate Analyses in All Analyzed Patients

because such a two dimensional cut-off algorithm can be used for validation experiments (see below). The two-biomarker cluster allocation of **figure 1B** was continued to be use for the clinical correlation studies.

Univariate analysis of clinicopathological parameters

Next, we were interested in comparing probability of loco-regional recurrence between the four clusters. As shown in **figure 2A**, the incidence of loco-regional recurrence was lowest for patients in cluster 3. The quantitative methylation pattern of cluster 3 patients was that the tumors showed significantly higher methylation at MINT3 and lower methylation at MINT17. This pattern corresponds with the clinically relevant rectal cancer patient cluster at high-risk for distant recurrence and decreased cancer-specific and overall survival that we previously identified¹⁸. Clusters 1, 2 and 4 showed similar probability outcomes. The difference in local recurrence probability became more evident and reached statistical significance after combining clusters 1, 2 and 4 ($P = 0.03$; **figure 2B**). This result shows that the specific combination of increased methylation at MINT3 and decreased methylation at MINT17 is predictive of reduced local recurrence probability. From this data it was demonstrated that quantitative methylation assessment of MINT3 and MINT17 identifies a patient group with an inversed risk incidence for distant recurrence and local recurrence.

Significance of MI differences between the cluster 3 and the combined clusters 1, 2 and 4 are given in **supplemental table 1**. It is demonstrated that the 95% confidence intervals of MINT3 and MINT17 do not overlap indicating that the rectal cancer patient groups' epigenetic classification is very distinct. The results also show that MINT1 and MINT2 can be helpful as discerning biomarkers for both patient groups.

Using univariate analysis, standard clinicopathological parameters including sex, age, TNM stage, N-status, tumor differentiation, location of distant recurrences, resection type and circumferential margin status were compared between cluster 3 and the combined group of clusters 1, 2 and 4. These parameters did not significantly differ between the two

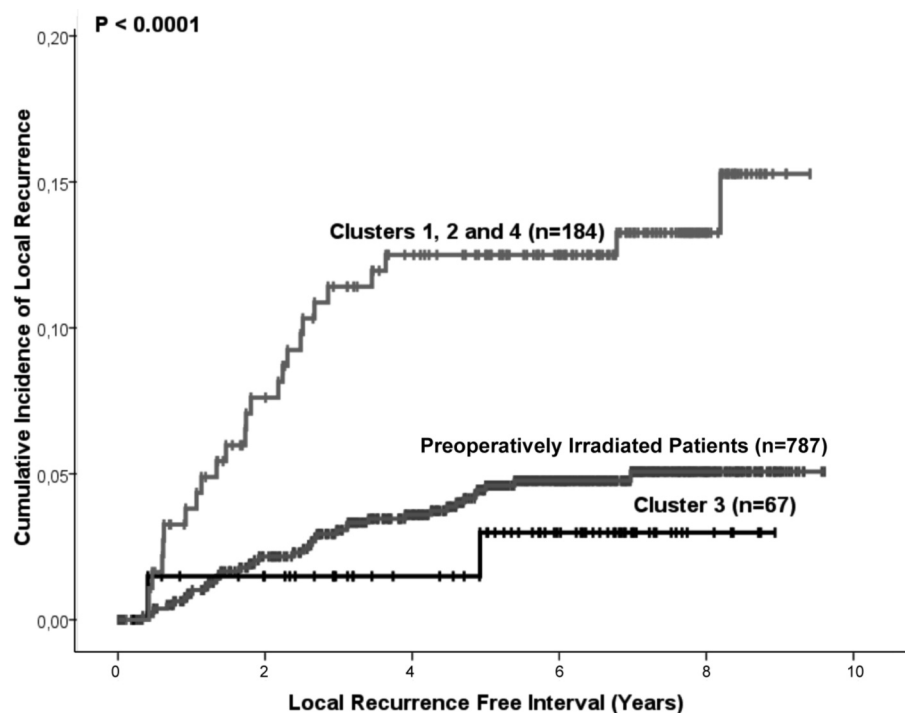


Figure 3. Cumulative incidence plots displaying local recurrence incidence differences between irradiated and non-irradiated patients. Cumulative incidence plot showing significant local recurrence incidence differences between patients that received preoperative radiation and patients belonging to the identified high-risk cluster for local recurrence.

patient groups identified based on methylation levels of MINT3 and MINT17 (**table 1**). In addition, no significant difference was observed for these standard clinicopathological factors between the patient groups when nodal status was taken into account (**supplemental table 2**).

Multivariate analyses

To assess whether the observed prognostic value of the clusters was independent of standard prognostic variables, we performed multivariate analyses. The Cox regression method was used to analyze standard prognostic factors of rectal cancer; T stage, N stage, circumferential margin status, distance of the tumor from the anal verge and tumor differentiation (**table 2**). Based on the epigenetic subclassification, the multivariate analysis showed patients of clusters 1, 2 or 4 to be at significant, over 10-fold, increased risk of local recurrence compared to cluster 3 patients. Nodal involvement was further significantly associated with increased local recurrence incidence and tumor involvement of the circumferential resection margin showed borderline significance.

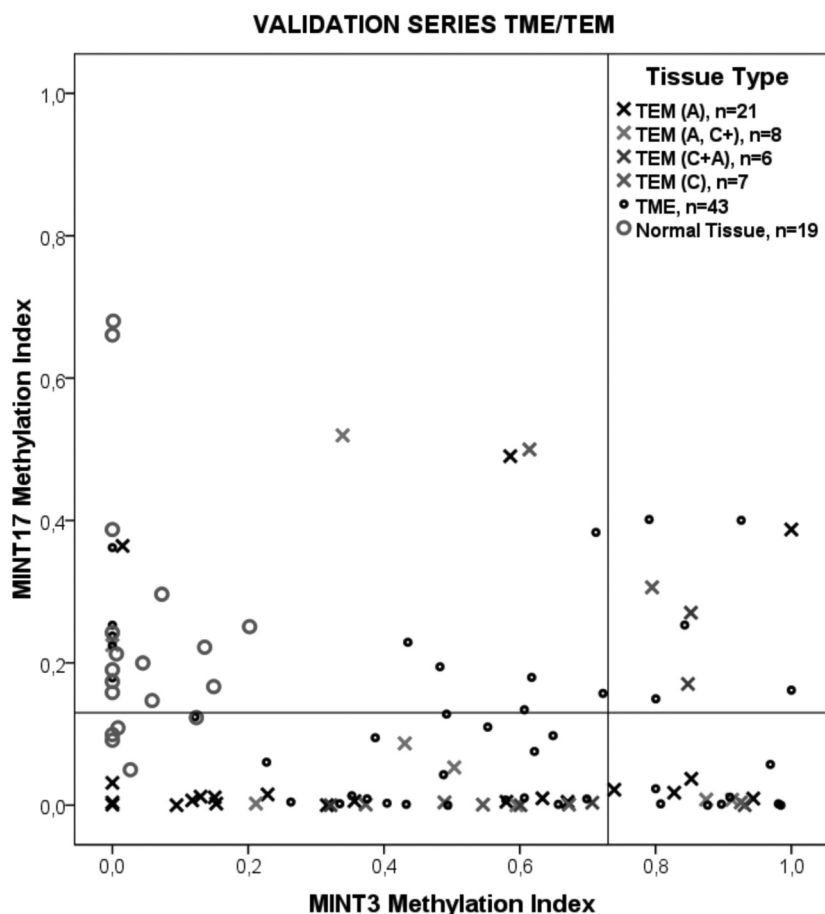


Figure 4. Two dimensional cut-off validation experiment results. Representation of MINT3 (X-axis) and MINT17 (Y-axis) methylation indices for patients of the TME trial, TEM study and 19 normal rectal mucosa samples. The horizontal and vertical reference lines represent the previously suggested cut-offs to separate the groups. For the TEM series, the following specimens were assessed: (A) adenoma with no carcinoma cells; (A, C+) adenoma with carcinoma cells; (C+A) carcinoma mixed with adenoma cells; and (C) carcinoma.

Comparison with preoperatively irradiated tumors

Because we found the non-irradiated cluster 3 patients to be at reduced risk for local recurrence, we were interested to see how recurrence probability rates of this group compared to those of preoperatively irradiated patients from the TME trial (**figure 3**). The irradiated patients were selected using the same clinical parameters that did not differ significantly from the non-irradiated selected patients (data not shown). The difference in loco-regional recurrence rate in cluster 3 patients was 3% versus 4.7% in patients who receive 5x5Gy before

surgery ($P=0.43$). Patients of high-risk clusters 1, 2 or 4 had significantly higher recurrence probability over time postoperatively compared to irradiated patients ($P<0.0001$). These results indicate that patients with TME alone identified by the quantitative two marker MINT profile have at least comparable, loco-regional recurrence rate compared to irradiated patients. This group will likely not be disadvantaged when preoperative radiotherapy before TME is not given and can be spared from unnecessary treatment morbidity.

External validation experiments

To test reproducibility of cluster allocation by the cut-offs established in **figure 1B** we measured MINT3 and MINT17 methylation levels in primary tumor tissues of 43 additional TME trial patients, and an independent group of 42 patients consisting of rectal adenomatous and cancer tissue from patients treated by TEM. In **figure 4** the results are given and show that 19%^{8/43} of TME and 19% of TEM patients were allocated to the prognostic cluster 3. The suggested cut-offs therefore show allocation of an independent group of rectal cancer patients, including patients with adenomas and very early disease stage, consistent in size and comparable to the size of the test group patients (27%). In the additional TME patient group there was only one event for local recurrence (which did occur in the high-risk cluster). The TEM group was treated differently and also contained premalignant stages and therefore not comparable. Further external validation of these clinical findings in future studies is needed.

To show the MI of the two biomarkers in normal rectal mucosa we added those data of 19 specimens to **figure 1B**. The results of the normal rectal tissue analysis shows that these are well separable from a cluster 3 patient which is important as these patients may be selected for treatment of their rectal tumor by surgery alone.

Discussion

Although recurrence rates have decreased to about 10% after the introduction of TME surgery, locally recurrent cancer remains an important clinical problem¹. In a previous study¹⁸, MINT methylation was shown to increase early during tumor progression, indicating that methylation of MINT loci is a factor acquired early during rectal tumorigenesis, and therefore can be used to subclassify early disease. Preoperative molecular profiling of the primary tumor could potentially be of great value to identify patients at high risk of developing local recurrence. This study shows that based on absolute quantitative methylation levels of the MINT3 and MINT17 loci, rectal cancers with a high-risk of local recurrence can be identified.

The MINT3 locus CpG island is localized on chromosome 1p34-35 just downstream of RBBP4 (retinoblastoma-binding protein 4)³², SYNC1 (encoding the syncoilin protein involved in cell cytoskeleton and extracellular matrix proteins)³³, YARS (tyrosyl-tRNA synthetase, involved in angiogenesis)^{34, 35} and s100p-binding protein (s100p is overexpressed in many solid tumors)^{36, 37}. MINT17 is localized on the long arm of chromosome 12, just upstream of the Harakiri (HRK) gene which is a member of the BCL2 gene family, which encodes apoptosis regulatory proteins and its expression in GI cancers is known to be regu-

lated by promoter region methylation³⁸. A regulatory or functional relation between the MINT3 and MINT17 CpG islands and the above mentioned genes has not been established. MINT locus methylation may therefore be seen as a surrogate biomarker. In the literature, several studies have shown clinical utility of the MINT loci methylation as predictive biomarkers in colon and gastric cancer, melanoma and renal cell carcinoma^{18, 39-42}. Our large-scale study adds novel findings that MINT locus methylation levels may have utility in rectal cancer local recurrence prediction. Importantly, in this study we propose an algorithm using only two MINT biomarkers with a simple cut-off and demonstrated validation of the algorithm to have excellent clustering capacity, even in early stage disease and that it separates cases well from normal rectal tissue.

This is the second study specific for rectal cancer from our group that shows clinical utility of MINT3 and MINT17 methylation levels. Strong probability differences between the two patient groups could be shown for local recurrence, with cluster 3 patients having an over 10-fold increased risk of developing local recurrences than patients allocated to the other clusters. Local recurrence rates of cluster 3 patients were comparable to those of irradiated rectal cancer patients and this shows that leaving out preoperative radiation can be done safely with the advantage of reducing treatment morbidity. This molecular stratification approach needs to be further investigated in a randomized multicenter trial to be validated.

Subdivision according to nodal status (data not shown) showed that patients in cluster 3 with a positive nodal status were at significant increased risk of local recurrence. Node negative patients in cluster 3, however, showed a significantly decreased cancer-specific and overall survival compared to the other clusters in our previous study¹⁸. This is explained by the fact that these node negative patients were found to be at significantly increased risk of distant recurrence in our previous study¹⁸. Early metastasizing of rectal cancer, in absence of evident nodal spread, may occur via the hematogenic route. This is supported by our group that circulating tumor cells can be detected in peripheral blood of early stage I/II colorectal cancer patients which has prognostic clinical utility⁴³. Our findings are in accordance with data from the TME trial showing that survival is determined predominantly by distant and not by loco-regional recurrence⁴. This suggests that non-locally recurrent and distantly spreading rectal cancer constitutes a separate subclass of rectal cancers which can be identified by our two-biomarker MINT methylation profile.

The TME trial showed reduction of rectal cancer local recurrence rates by adding preoperative radiotherapy⁷. A reduction of distant recurrence of rectal cancer, as well as improved disease-free and overall survival, has been shown in several randomized controlled trials, either alone or in combination with radiotherapy⁴⁴⁻⁴⁶ using adjuvant chemotherapy. This is the first study to demonstrate that loco-regional recurrence patterns of AJCC stage I, II and III rectal cancers can be distinguished using preoperatively assessable, quantitative epigenetic subclassification of primary rectal tumor tissue. Our previous study showed the methylation status of the described MINT loci to have utility in predicting distant recurrence probability in stage I and II disease. Therefore, a new treatment stratification approach for rectal cancer can be suggested as follows: after preoperative assessment of primary tumor biopsy specimens of MINT3 and MINT17 methylation levels, about 30% of patients could be spared from preoperative radiation therapy, but might benefit from systemic tre-

atment. The other 70% should receive preoperative radiotherapy, and if node-positive, post-operatively systemic treatment can be considered. The identification of patients who do not need pre-operative radiotherapy would likely reduce long-term morbidity and improve quality of life⁴⁷. The results of our study should be further evaluated in order to improve planning of therapeutic regimens for rectal cancer patients in the preoperative phase.

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CHAPTER 8

Development of Sporadic Microsatellite Instability in Colorectal Tumors Requires Hypermethylation at Methylated-IN-Tumor Loci in Adenoma

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Abstract

Background: Microsatellite instability (MSI) and genomic hypermethylation of methylated-in-tumor (MINT) loci are known strong prognostic indicators of a subgroup of sporadic colorectal cancer (CRC) patients. A study was designed to determine whether methylation of MINT loci during adenoma progression to CRC is related to MSI in CRC.

Methodology: Methylation index (MI) was assessed by absolute quantitative assessment of methylated alleles (AQAMA) at seven MINT loci in CRC and contiguous adenomatous and normal tissues of 115 patients using in situ histopathology analysis. Results were then validated in primary tumor tissue from an independent group of 54 CRC patients.

Results: Increased methylation levels of MINT loci 1 and 31 were significantly associated with MSI and shown to be adenoma-specific. The total MI and number of methylated loci were 3-fold ($P=0.02$) and 5-fold ($P=0.004$) higher, respectively, in adenomas associated with microsatellite stable (MSS) versus MSI-High primary CRC. MINT methylation levels correlated with mismatch repair protein expression, MSI, B-RAF(V600E) mutation status, human mut-L homologue 1 (*hMHL1*) methylation status and CRC survival in an independent patient group.

Significance: Methylation levels of specific MINT loci were prognostic indicators of colorectal adenomas that will develop into sporadic microsatellite instable CRCs. The study indicates that increased MINT locus methylation precedes MSI in absence of malignant invasive tumor features and may have clinical pathology utility.

Introduction

Epigenetic changes in epithelial cells, such as DNA methylation of CpG islands, have been related to the genesis and progression of some gastrointestinal (GI) cancers^{1, 2}. Aberrations in DNA methylation are considered to be as important as genetic alterations in GI tumor initiation and progression. In colorectal cancer (CRC), both hypomethylation and hypermethylation of promoter-region related CpG islands have been correlated with clinical and pathology parameters^{3, 4}. A number of tumor-related gene promoter regions have been demonstrated to be methylated in early dysplastic stages of the colonic malignant pathway such as in hyperplastic polyps⁵, aberrant crypt foci^{6, 7} and adenomas⁸⁻¹⁰. Progressive genomic and epigenomic aberrations may be linked in CRC¹¹. For instance it has been reported that widely increased methylation in sporadic CRCs overlaps with microsatellite instability (MSI)¹²⁻¹⁵. Studies have been performed to pinpoint the onset of MSI in hereditary nonpolyposis colorectal cancers (HNPCC) that show germline mutations (mt) in mismatch repair (MMR) genes^{13, 14, 16, 17}, but as yet no study has examined this process in sporadic CRC. Further, the relation between genomic instability and epigenomic aberrations during sporadic CRC progression is not clear.

We recently described on-slide sodium bisulfite modification (SBM) technique for gene methylation analysis in small (1-2 mm²) tissue areas isolated from a single section of paraffin-embedded archival tissue (PEAT)¹⁸. On-slide SBM allows comparison of gene methylation in the primary CRC and a contiguous adenoma lesion within the same tissue section. Genomic aberrations such as MSI and epigenomic changes can be tested at the same time from the same microdissected tumor lesion. Simultaneous assessment of CRC, precursor and normal tissues in the same PEAT section would provide valuable information that has not been available in the past.

In a previous study, our group assessed methylated-in-tumor (MINT) loci 1, 2, 12 and 31 and demonstrated technical feasibility of detecting methylation level differences between different colorectal tissue areas within the same tissue section using these loci⁸. MINT loci are conservative human genomic sequences that adhere to the CpG island definition,^{19, 20} found in non-coding genomic regions, and gene regulatory or other functional attributes to date are unknown. We have previously shown the clinical utility of methylation levels of specific MINT loci in rectal cancer²⁰ and melanoma⁴ and other groups have studied MINT locus methylation in colorectal²⁰⁻²² and gastric cancers^{23, 24}. Methylation of MINT loci has also linked to MSI in CRC^{25, 26}; however this event has not been studied in adenomas. Therefore, we examined the methylation level of seven MINT markers in cancer, precancer, and normal tissue.

The objective was to identify if MINT locus hypermethylation influences MMR during early stages of CRC development. Methylation at MINT loci was quantified using the on-slide SBM technique combined with AQAMA. We further assessed MSI status, K-RAS mt at codons 12 and 13, B-RAF V600E mt, and methylation status of the MMR gene, *MutL homolog 1 (hMLH1)*, in primary CRC as well as the contiguous precursor lesion in order to rigorously assess whether these events occur at early stages of CRC development. Our *hypothesis* was that adenoma tissue adjacent to MSI-high (MSI-H) invasive CRC tissue has increased MINT locus methylation levels, methylation of the *hMLH1* promoter region, and

MSI in comparison to precursor cancer lesions adjacent to microsatellite stable (MSS) cancers. We then validated in an independent patient group that MINT methylation levels positively correlated to *hMLH1* methylation index (MI), MSI-status, B-RAF V600E mt, MMR protein expression, and disease survival.

Materials and Methods

Patient Specimens

For the first phase of the study, we searched the cancer registry database at Saint John's Health Center (SJHC) to identify patients whose resected CRC specimen contained histopathologically confirmed areas of adenoma as well as invasive cancer. Excluded were any cases without available PEAT specimens. Consecutive patients were identified in reverse chronological order until an adequate sample size was reached. The final cohort of 115 patients underwent surgical resection of CRC between 1996 and 2009.

A single H&E section was prepared and mounted and 7- μ m sections were consecutively cut and mounted on silane-coated glass slides for DNA studies. Areas of adenoma (serrated and non-serrated), carcinoma and normal tissue as well as the adenoma type were identified by a surgical oncology pathologist (R.R.T.) with expertise in CRC pathology^{8, 27}.

Archived CRC primary tumor tissue PEAT blocks collected from a cohort of 54 patients that underwent surgical tumor resection at the Leiden University Medical Center (LUMC) between 1990 and 2001 were used for the validation study. All patients had been previously analyzed for MMR sufficiency at LUMC's pathology department. From each PEAT block, a single section was cut for HE staining, and 7- μ m sections were consecutively cut on coated slides for on-slide SBM. Tumor areas were identified and marked by an expert CRC pathologist (H.M.). Study protocols were approved by the institutional review boards at the LUMC and at Saint John's Health Center.

AQAMA and *hMLH1* Methylation Assessment

DNA from PEAT was modified *in situ* by sodium bisulfite according to our previously reported protocol¹⁸. AQAMA of MINT loci 1, 2, 3, 12, 17, 25 and 31 was performed and data analyzed as previously described^{8, 28}. *hMLH1* methylation status was analyzed by capillary-array-electrophoresis methylation-specific PCR (CAE-MSP)^{27, 29, 30}. Primer sequences were those whose correlation with *hMLH1* protein expression had been validated in previously reported studies³¹⁻³⁵.

MSI, K-RAS and B-RAF mt Analysis

For MSI assessment in the first phase study, an additional tissue section from each of the specimens was deparaffinized and stained with hematoxylin to identify tumor cells for DNA isolation, as previously described⁸. Normal epithelial cells were harvested either from a separate tissue block of the same specimen (i.e. from uninvolved resection margins). As markers for MSI assessment, we used three quasi-monomorphic mononucleotide repeats BAT25, BAT26 and BAT40 and two microsatellite, dinucleotide repeats D2S123 and D5S346 according to the revised Bethesda guidelines³⁶. Forward primers (**supplementary table S1**) were

PCR Primer Sequences for MSI Assessment		
	Forward Primers	Reverse Primers
BAT25	5'-CCTCGCCTCCAAGAATGTAA-3'	5'-TGCTTTTGGTTACCACACTTCA-3'
BAT26	5'-GCAGTCAGAGCCCTTAACCTT-3'	5'-CCATTTAAAGCTAGTTATCTAATCCA-3'
BAT40	5'-AAGATTAACTTCTACACCACAACC-3'	5'-GTAGAGCAAGACCACCTTGT-3'
D2S123	5'-TGGCCAGAGAAATTAGACACA-3'	5'-TCTGACTTGGATACCATCTATCTATCT-3'
D5S346	5'-TTCAGGGAATTGAGAGTTACAGG-3'	5'-TCACTCTAGTGATAAATCGGGAAA-3'

Supplementary Table S1

dye-labeled for automated high-throughput multiplex detection by CAE³⁷ (CEQ 8000XL, Beckman Coulter, Fullerton, CA). PCR product fragment length differences among different tissue categories were visualized by the CEQ software (Beckman Coulter). PCR products from the five amplified microsatellite regions in adenoma and cancer were compared to the reference normal epithelium. K-RAS (codons 12 and 13) and B-RAF (V600E) mt were assessed by a peptide nucleic acid (PNA) clamp- and locked nucleic acid (LNA) probe-based quantitative real-time PCR assay as previously described^{38, 39}. These assays were performed in triplicate and carried out at least twice to confirm accuracy. A gene in a specimen was considered mutated when results were uniformly positive in the triplicates under the optimal conditions. Respective normal and positive PEAT and cell lines controls were run in each assay.

Second Patient Group MMR and mt Status Analysis

The diagnostic techniques used by the LUMC's pathology department for MSI status and MMR protein expression assessment were as described⁴⁰. Briefly, MSI-status was assessed by MSI Analysis System (Promega Corp.; five mononucleotide and two pentanucleotide repeats)⁴¹. Immunostaining of MMR proteins was performed with anti-MLH1 (clone G168-728; 1:50; BD Biosciences, NJ) and anti-PMS2 (clone A16-4; 1:50; BD Biosciences). IHC staining was performed on 4-µm-thick, PEAT sections from tissue microarray (TMA). IHC staining patterns of these MMR proteins were evaluated using normal epithelial, stromal, or inflammatory cells, or the centers of lymphoid follicles as internal controls as previously published⁴². TMAs contained three cores punched from each case's primary tumor. Individual cores of the TMA were scored as either positive (showing nuclear staining in at least some tumor cells) or negative. Patients were considered positive if at least one TMA tissue core showed nuclear staining and negative otherwise. Cases in which both tumor and internal control stained negative were not included in the study. Cases were scored by two independent reviewers (H.M. and N.F.C.C. de M.) and in case of a tie, both reviewers reassessed the slides for consensus. K-RAS⁴³ and B-RAF⁴⁴ mt were detected by means of sequencing as previously described.

Biostatistical Analyses

Significance of changes in MI at individual MINT loci and total MI was evaluated with non-parametric tests for related and independent sample sets (Wilcoxon's rank-sum test, Mann-

	Total Patient Group (n=115)	Patients with Cancer Tissues (n=79)	Micro- satellite Instable (n=9)	Micro- satellite Stable (n=70)	P-value
Characteristics					
Sex					
Male	45 (39%)	35 (44%)	3 (33%)	33 (47%)	0.44
Female	70 (61%)	44 (56%)	6 (66%)	37 (53%)	
Age					
Mean (SE)	76.1 (1.2)	75.0 (1.0)	81.4 (7.3)	76.1 (10.6)	0.15
Tumor Location					
Cecum	24 (21%)	16 (20%)	3 (33%)	14 (20%)	0.03
Colon Ascendens	15 (13%)	9 (11%)	4 (44%)	5 (7%)	
Hepatic Flexure	6 (5%)	4 (5%)	0	4 (6%)	
Colon Transversum	17 (15%)	12 (13%)	2 (22%)	9 (13%)	
Colon Descendens	4 (4%)	3 (4%)	0	3 (4%)	
Sigmoid Colon	13 (11%)	9 (11%)	0	8 (11%)	
Rectosigmoid	12 (10%)	9 (10%)	0	9 (13%)	
Rectum	24 (21%)	17 (22%)	0	18 (26%)	
Adenoma Type					
Sessile Serrated	9 (8%)	5 (6%)	2 (22%)	3 (4%)	0.10*
Classic:	106 (92%)	74 (94%)	7 (88%)	67 (96%)	
-Villoglandular	7 (6%)	4 (5%)	0	4 (6%)	
-Tubular	11 (10%)	8 (10%)	2 (22%)	6 (9%)	
-Tubulovillous	15 (13%)	10 (13%)	1 (11%)	9 (13%)	
-Villous	73 (63%)	52 (66%)	4 (44%)	48 (68%)	
*Fisher’s exact test evaluating differences of MSI status in sessile vs classic adenoma in patients with adenoma as well as carcinoma available for analysis (n=72)					
Table 1: Patient and tumor characteristics.					

Whitney's u-test). Fisher's exact test (two-tailed) was used to assess significance of differences between *hMLH1* methylation prevalence among groups.

Spearman's rank correlation coefficient between quantitative MINT MI and *hMLH1* MI was assessed as a non-parametric measure of correlation. Correlations with clinical parameters were tested with Pearson's χ^2 -square and in case of ordinal variables with Mann-Whitney's or Kruskal-Wallis' u-tests. Postoperative distant recurrence probability disease-free and overall survival over time were visualized with Kaplan-Meier plots and significance was assessed with the log-rank test. Cox's regression models considered the following variables that were entered in a stepwise manner: age, N-stage, MSI status, tumor differentiation. $P < 0.05$ (two-sided) was considered significant. SPSS (SPSS Inc., Chicago, IL) statistical software version 16.0.1 was used for all analyses.

Results

MINT Locus Methylation Status and MSI during CRC Development

In the first phase of the study, we assessed operative specimens from 115 patients who underwent open resection of CRC whose specimen, according to the pathology report contained adenoma as well as cancer (see **table 1** for patient characteristics). We first reviewed the newly cut sections for our study. Adenoma cases with high-grade dysplasia were not selected to obtain good separation between the adenoma and carcinoma category, and only carcinoma tissue with evidence of at least submucosal invasion was selected. Subsequently, 27 specimens were deleted. Of the remaining 88 specimens that contained low- or medium-grade adenomatous dysplasia, 79 also had invasive carcinoma tissue. 50 of these specimens had normal tissue present.

MSI status was analyzed using five established genomic markers³⁶ in normal and cancer tissue of 79 patients. Nine patients (11%) showed instability in ≥ 4 biomarkers and were classified as MSI-H. Sixty-five patients (83%) did not show a shift in any of the markers, and five (6%) patients had a single dinucleotide repeat affected. These 70 patients with ≤ 1 aberrant MSI-marker were classified as MSS. The median age of MSI-H patients was 81.4 yrs (range 69-91). **figures 1** and **2** show a representative PCR product analyses of AQAMA and CAE.

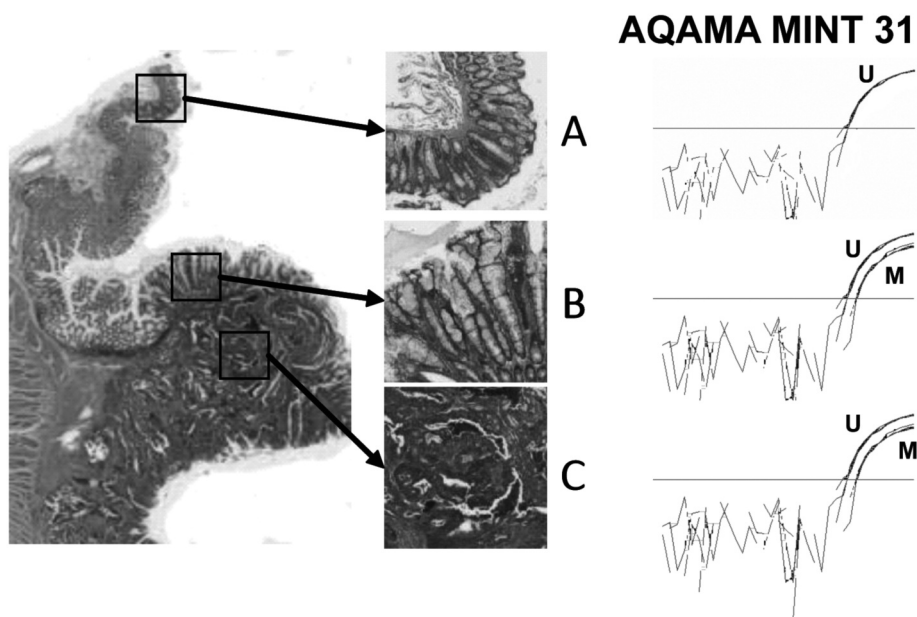


Figure 1: Representative examples of molecular analyses in normal (A), adenoma (B) and cancer (C) tissue. AQAMA (MINT31) real-time PCR results showing results in triplicate of exponential amplification of methylated (M) and unmethylated (U) dye probe signal. Y-axis represents signal intensity, X-axis represents PCR cycle number.

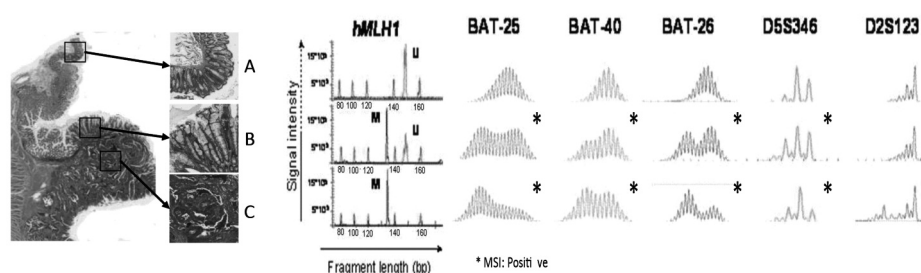


Figure 2: Representative examples of molecular analyses in normal (A), adenoma (B) and cancer (C) tissue. CAE results of amplified dye-labeled PCR products of 5 MSI biomarkers and *hMLH1* methylation-specific (M, in blue) and unmethylated-specific sequence (U, in green) primer sets. Red peaks represent the DNA ladder signal. The horizontal axis represents size of the PCR product in base-pairs (bp). The vertical axis represents arbitrary units of signal intensity of detected amplicons.

We compared MIs of the three tissue categories (normal, adenoma, cancer) for each individual MINT locus and divided the cases into MSI-H and MSS (**figure 3**). Methylation levels of MINT loci 1, 2, 3, 12 and 31 were significantly higher in CRC than in normal epithelium. Methylation levels of MINT loci 1, 2, 12 and 31 were significantly higher in MSI-H CRC compared to MSS CRC. Methylation levels of MINT loci 1 and 31 were significantly higher in adenomas contiguous to MSI-H CRC than in adenoma contiguous to MSS CRC. The total MI of tumor-specific, MSI-related MINT loci 1, 2, 12 and 31 was 4.5 times higher in adenomatous tissue paired to MSI-H tumors ($P=0.02$, **figure 4**). The average number of tumor-specific MSI-related MINT loci showing $MI>0.1$ was $1.5 (SD\pm 1.4)$ in MSI-H related adenomas versus $0.3 (SD\pm 0.6)$ in MSS related adenomas ($P=0.004$, **figure 4**). MINT17 MI was MSI-related in CRC but was also present in normal tissue. MSI status was significantly correlated to right-sided tumor location and not to serrated polyp type (**table 1**). MI was significantly higher in the serrated adenomas only at MINT1 and MINT31 ($P=0.002$ and $P=0.02$, respectively) compared to non-serrated adenomas. There were no significant MINT methylation level differences between carcinoma tissue contiguous to serrated adenomas versus carcinoma tissue contiguous to classic adenoma. Methylation levels of the two serrated/MSI-H adenoma were relatively low compared to the non-serrated/MSI-H adenoma.

From this we conclude that increased methylation levels of MINT loci 1, 2, 12, 17 and 31 are markers of MSI-H sporadic CRCs and except MINT 17 these are tumor-specific events. Increased methylation of MINT 1 and 31 were identified as adenoma/MSI-specific events and these loci are differentially methylated in sessile serrated adenomas compared to non-serrated adenomas. The MSI-H precursor lesions identified by MINT loci methylation analysis did not show any distinct histopathologic features. The total MIs and number of affected biomarkers of the cancer/MSI-specific MINT loci show significant differences and are indicators of colorectal adenomas that will become a microsatellite instable CRC.

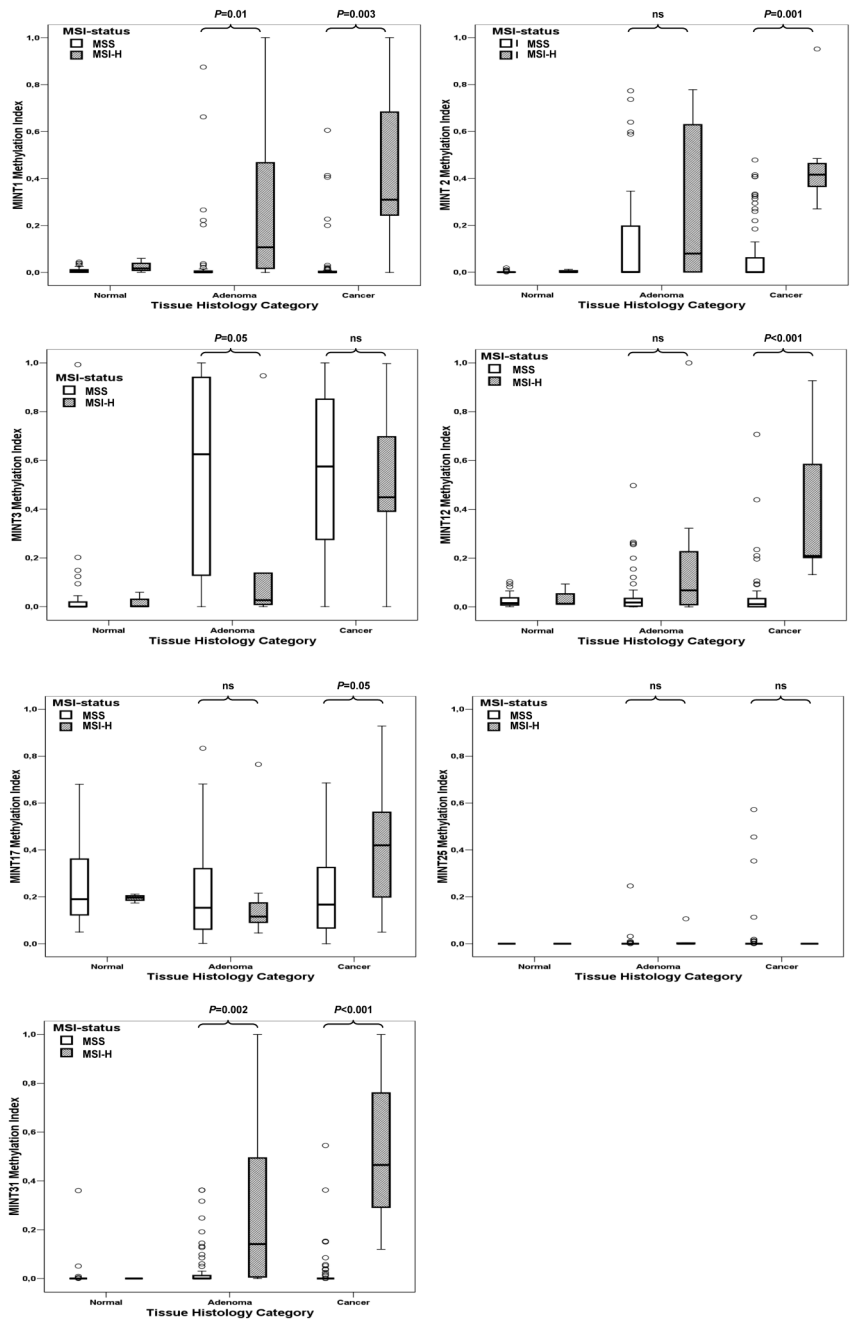


Figure 3: Box plots summarizing MI values for normal, adenoma and cancer tissue separated into MSS and MSI-H tumors for each individual MINT locus.

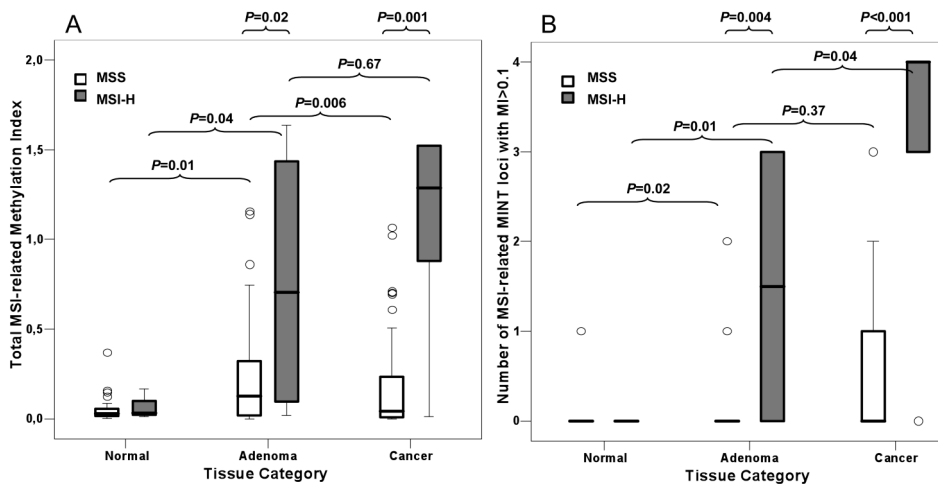


Figure 4: Box plots representing normal, adenoma and cancer tissue separated into MSS and MSI-H tumors. (A) Y-axis represent total MI of 4 MSI-related MINT loci (MINT1, 2, 12 and 31). (B) the Y-axis represents the number of MSI-related MINT loci with $MI > 0.1$.

***hMLH1* methylation status and MSI during CRC development**

Subsequently we determined whether the relation between MINT methylation levels and MSI in colorectal precursor lesions can be explained by a relation to methylation of the *hMLH1* MMR gene. Therefore, *hMLH1* methylation status was assessed in normal, adenomatous and cancer tissues of the nine MSI-H patients and in a control group of 13 MSS patients of which normal, adenomatous and cancer tissues were available of the first phase study. All nine MSI-H patients' tumors showed methylation of *hMLH1* compared to 2 of 13 of the MSS controls ($P < 0.001$). Six of 9 MSI-H adenomas showed methylation of *hMLH1* compared to 2 of 13 adenomas paired to the MSS controls ($P = 0.02$). In 6 MSI-H patients with high tumor MI (≥ 0.88), instability of at least four MSI-biomarkers could be shown in only three of six (50%) of the associated adenomas. The paired adenoma tissue of these six patients did show increased MI at the MSI-specific MINT loci (≥ 0.56) compared to their paired normal epithelium. Together, MI of MINT1, 2, 12 and 31 and collateral methylation of *hMLH1* constitute specific biomarkers of adenomatous colorectal tissue that shows MSI or will develop into a sporadic MSI-H CRC. The results further demonstrated that MINT locus methylation along with *hMLH1* methylation precede MSI in the sequence of CRC development before histopathology signs of cancerous invasiveness are present.

B-RAF and K-RAS mt during CRC development

Studies in CRC have shown correlations between increased methylation of important tumor-related genes and mt of B-RAF (V600E) and K-RAS (codons 12 and 13), often in combination with MSI-H^{15, 19, 45, 46}. The significance is not clearly understood, however it has been suggested that these events synergistically induce a high-risk phenotype that results in a clinically dis-

tinct subtype of CRC. We determined B-RAF V600E and K-RAS mt in the same group used for *hMLH1* methylation status analysis (9 MSI-H cases and 13 MSS cases): the mt incidence was 46% and 18% for K-RAS and B-RAF, respectively. B-RAF mt was shown in 44% of the MSI-H carcinomas and in 63% of the contiguous adenomas. By comparison, none of the MSS adenomas and carcinomas had B-RAF mt ($P=0.003$ and $P=0.02$, respectively). K-RAS mt was more frequent in MSS adenomas and carcinomas (54% and 62% respectively), compared with MSI-H lesions (13% and 22%, respectively), although this did not reach statistical significance ($P>0.09$). These results were as expected based on previous reports. Total methylation levels at MINT loci 1, 2, 12 and 31 were significantly increased in B-RAF mt adenomas ($P=0.03$) as well as B-RAF mt carcinomas ($P=0.002$). None of the MINT loci were differentially methylated in K-RAS mt versus wild-type adenomas or carcinomas. This assessment demonstrated that genetic and epigenetic events synergize in the earliest phase of CRC development.

Quantitative Methylation and Mismatch Repair

The methylation levels of five MINT loci dispersed were related to methylation of *hMLH1* and to MSI. These findings were validated using a second independent cohort of CRC patients whose *hMLH1* expression was known. We first corroborated whether increased MINT MI linearly correlates to *hMLH1* MI, and subsequently whether it affects MMR at the protein level. MIs at the MSI-associated MINT loci (1, 2, 12, 17, 31) and *hMLH1* MI were measured in 54 CRC specimens that were previously analyzed for MMR sufficiency. Two outcome parameters were analyzed: total MI (defined by the sum of MIs of MINT loci 1, 2, 12, 17 and 31) and number of methylated MINT loci. The number of methylated MINT loci in a specimen was determined as the number of MINT loci (1, 2, 12, 17 and 31) that exceeded the MSI-H related MI cut-off. The average MI + 1SD of MSS CRC specimens from the first phase study determined this cut-off for MSI-related methylation. Correlation analysis showed that both outcome parameters were significantly associated with *hMLH1* MI with a correlation coefficient of $\rho=0.43$ ($P=0.002$) and $\rho=0.400$ ($P<0.001$) for total MI and number of methylated MINT loci, respectively. Furthermore, in **Table 2** the results for the association of *hMLH1* protein expression in relation to MI are given. *hMLH1* underexpression

	<i>hMLH1</i>				
	Number of Patients	Average Methylation Index (SD)	P	IHC non- expressing*	P
Number of MINT	0	32	0.14 (0.22)	5 (16)	0.002
loci methylated	1	7	0.08 (0.10)	2 (29)	
	2	4	0.01 (0.01)	1 (25)	
	3	5	0.72 (0.34)	1 (20)	
	4	2	0.42 (0.25)	2 (100)	
	5	4	0.43 (0.37)	4 (100)	

* Confirmed by underexpression of PMS2

Table 2: Relation of Methylated Loci and MI to *hMLH1* Methylation Status and Protein Expression

was corroborated by underexpression of its co-protein PMS2 in all cases⁴⁷. Examples of IHC and CEA-MSP results are given in **figure 5**. Subsequently all MMR protein deficient cases showed MSI by PCR analysis. Again we analyzed two outcome parameters: number of affected MINT loci and total MI. These results demonstrated a linear relation of quantitative MINT methylation to *hMLH1* down regulation and subsequent MMR deficiency, and importance of both the number of loci involved as well as total MI.

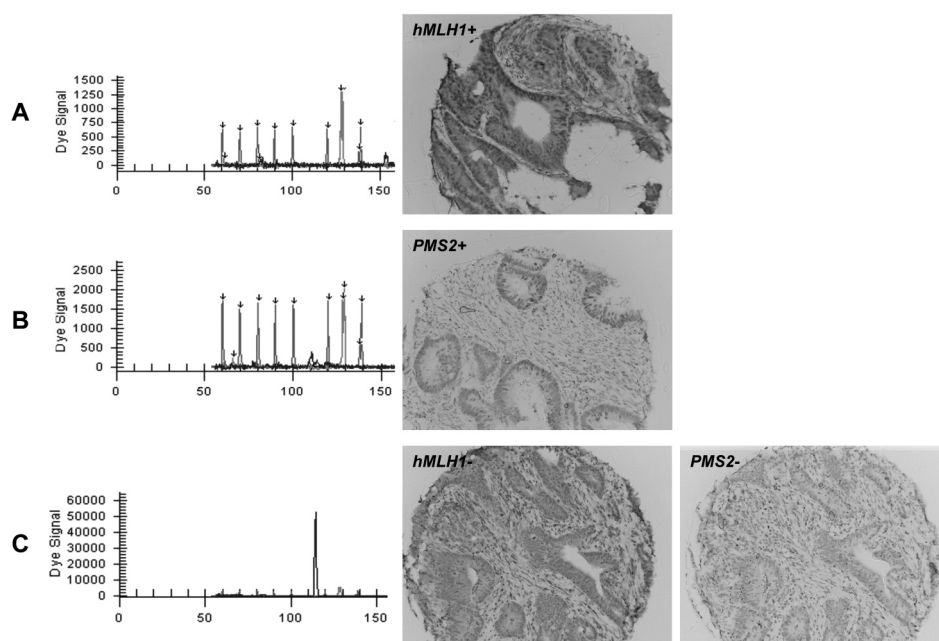


Figure 5: Representative IHC staining of *hMLH1* and *PMS2* proteins. In **A** and **B** examples of *hMLH1* and *PMS2*, respectively, expressing CRC with their corresponding CAE-MSP results for *hMLH1* showing unmethylated signal only. In **C**, an example of a CRC showing methylation of the *hMLH1* promoter region with corresponding absence of nuclear staining of *hMLH1* which is confirmed by underexpression of *PMS2*.

B-RAF and K-RAS mt were also assessed in this patient group. B-RAF mt cases (n=11, 20%) were significantly associated with MSI-H ($P=0.003$) and under expression of *hMLH1* ($P=0.003$). K-RAS mt tumors (n=14, 26%) did not show any significant associations with mismatch repair parameters. B-RAF mt tumors had significantly increased methylation at MINT1, 2, 12, 17 and 31 ($P<0.001$, $P<0.001$, $P=0.001$, $P<0.001$ and $P=0.003$, respectively). None of the B-RAF mt tumors had K-RAS mt. Methylation levels did not differ significantly between K-RAS mt tumors and K-RAS wild-type tumors.

Clinical outcome in our validation group was analyzed with respect to the number of MINT loci that showed MSI-related methylation. The result (**figure 6**) confirmed previous reports of a direct correlation between methylation levels recurrence-free survival.

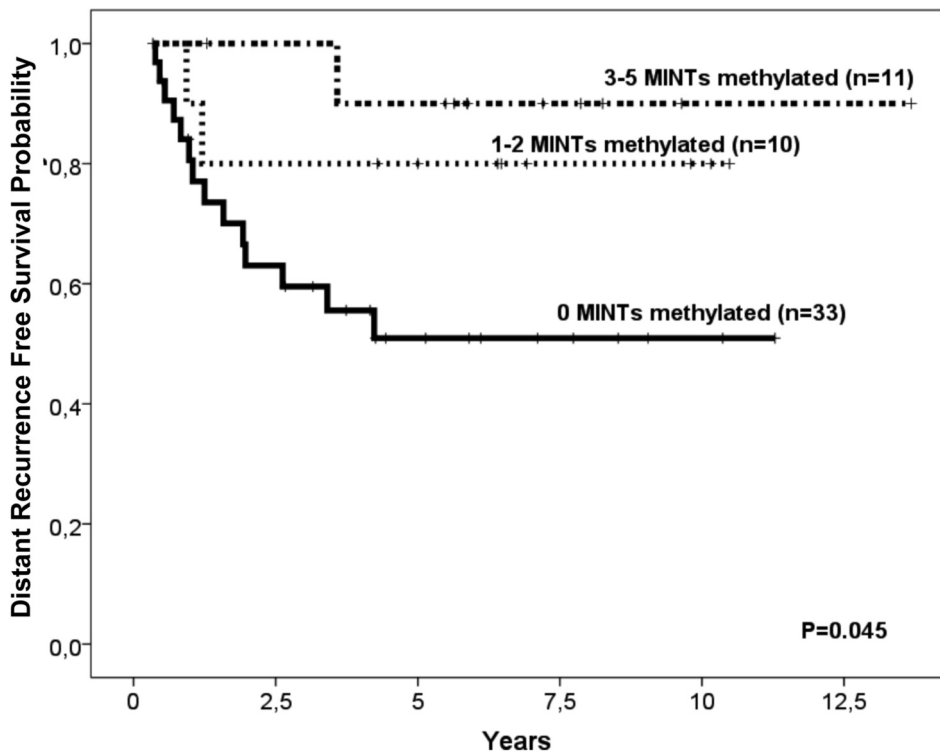


Figure 6: Kaplan Meier curves for distant recurrence probability stratified into patients 0, 1-2 or 3-5 MINT loci with MSI-related methylation.

Multivariate analysis showed that number of methylated, MSI-related MINT loci was an independent predictor of distant recurrence-free survival (HR 0.20, 95% CI 0.04 - 0.96, $P=0.02$), disease-free survival (HR 0.33, 95%CI 0.12 - 0.87, $P=0.04$) and overall survival (HR 0.38, 95%CI 0.14 - 1.00, $P=0.05$) along with nodal stage, and patient age.

Discussion

Epigenetic silencing of *hMLH1* has been identified as the causative mechanism for MSI in non-hereditary CRC by several groups^{13-15, 48}. Our study adds important findings in genomic and epigenomic events at an early stage of CRC development. We could demonstrate that the relation between MSI and methylation is present at the premalignant stage in colorectal tissue without cancer histopathology. MI as well as the number of MINT loci methylated was significantly increased in MSI-H versus MSS CRC tumors and importantly, this was also found in their precursor lesions. In adenomas associated with MSI-H tumors there was subsequently significantly more hypermethylation of *hMLH1* detected. This suggests

that CRC precursor lesions with a higher MI at MINT loci may be more likely to have the *hMLH1* MMR gene methylated as well. This has not been reported to date. We were able to demonstrate this by combining on-slide SBM and AQAMA techniques on PEAT which allowed accurate comparison of MIs between CRC tissues and contiguous adenoma tissues. Previous approaches reported were not capable of accurate analysis based on comparable synchronous histopathology and depended on comparison of specimens of different stages of CRC formation from different patients. The study's results demonstrate significant changes in epigenetic and genetic markers at the origin of the development of a subset of CRC tumors.

The relation between degree of MINT methylation and MSI was further validated on the MMR protein level in a second independent patient group. MINT MI linearly correlated with *hMLH1* MI, and subsequently resulted in underexpression of *hMLH1* protein and MSI. This suggests that deregulation of a CRC's MMR system causing sporadic MSI results from the increased likelihood of methylation of *hMLH1* in a tumor with increased MIs at MINT loci. This demonstrated the *level* of DNA methylation to be an underlying mechanism of sporadic MSI and that this accumulation of DNA methylation affects the MMR system early during CRC development. We corroborated the validity of the quantitative MINT methylation AQAMA assay further by reproducing prognostic value which previous studies have shown in colorectal and gastric cancer⁴⁹⁻⁵¹.

Recently some studies on DNA hypermethylation in CRC include B-RAF and K-RAS mt analyses. B-RAF mt is more frequently found in sporadic CRCs showing MSI-H (approximately 50%), CIMP+ and *hMLH1* methylation^{52, 53} and are possibly associated with adverse disease survival⁵¹. Specific single-nucleotide mt of K-RAS have predictive value in stage III/IV CRC, likely related to systemic treatment response⁵⁴⁻⁵⁶. However, as yet no study has demonstrated a functional relation between MMR impairment and the reduced cell signaling properties of K-RAS or B-RAF mutant CRCs. Our results confirm the association of the V600E B-RAF mt to MINT locus hypermethylation and add that this ensemble of events initiates early in CRC development in non-serrated adenomas with only low or intermediate dysplasia.

Early identification of patients at risk for developing MSI-H phenotype sporadic cancers by AQAMA of MINT loci may be of clinicopathology utility. An approach that may be useful in the future would be to examine whether other polyps collected during subsequent colonoscopies from the same patient have similar DNA methylation results on repetitive samplings; this may identify a high-risk predisposition to CRC development. The polyp recurrence probability of patients with adenomas with high MINT locus methylation could be studied and the frequency of follow-up colonoscopies may be adjusted for such patients. Our results further show that MINT loci methylation levels can discriminate normal from adenomatous tissue. The MINT biomarkers could also be a part of a screening panel tested on fecal DNA to identify patients that should undergo colonoscopy. Not only MSI-H precursor lesions may be identified by MINT biomarkers 1, 2, 12 and 31 but also MSS lesions may be identified by MINT3 methylation. Furthermore, it is known that MSI-H cancers respond differentially to common chemotherapeutics. For instance, irinotecan is suggested to be more effective than 5-FU in these tumors. With the development of new targeted drugs, preventive treatment regimens may be indicated for high-risk patients with adenomas that

show high MINT locus methylation. Also there is increasing evidence for MSI-induced generation of novel tumor-specific carboxy-terminal frameshift peptides (FSPs) in MSI-H cancers⁵⁷. Patients who have polyps with high MINT locus methylation may become a stratified target patient group for vaccination trials of FSP-based approaches.

This is the first study to demonstrate a correlation between MSI and MINT hypermethylation in CRC precursor lesions, and this may be attributed to suppression of the MMR system by progressive epigenetic events. Therefore, the *degree* of genomic hypermethylation in CRC precursors is implicated as a potential causative factor of sporadic MSI CRC. Specific MINT locus methylation identification may have utility in early identification of colorectal polyps that will develop into sporadic MSI-H cancers.

References

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General Discussion and Future Perspectives

General Discussion

The treatment of GI cancers and in particular that of gastric, colon and rectal cancer is multidisciplinary and requires close collaboration between surgeons, gastroenterologists, radiologists, pathologists, radiation therapists, medical oncologists, oncologic nurses and psychologists. Surgical tumor removal is the mainstay of GI cancer treatment. The surgeon therefore plays a central role in coordinating the various treatment options after the initial diagnosis. Biomarkers may improve guiding of such treatment decisions. Postoperatively, after complete pathologic disease staging, the current markers in GI cancer (i.e. pTNM stage, CT/PET-imaging) are adequate and remain our best tools in the shed. Patient disease recurrence and survival data, however, indicate that there is much room for improvement in GI cancer staging, especially for early disease patients¹⁻³. Also, there is a need for biomarkers that can guide preoperative treatment. One must keep in mind that TNM-staging is an observer-dependent, anatomical and therefore surrogate marker of cancer that does not include primary cancer cell properties. Hence, there is a need for a new category of GI cancer biomarkers. Primary tumor cell molecular features, assessed in preoperative biopsy material, are a very attractive tool which is currently under investigation. Molecular oncologic scientists are increasingly appreciating the role of epigenetic factors in GI carcinogenesis. Epigenetic primary tumor biomarkers, such as DNA methylation therefore merit evaluation to assess their possible utility to aid GI cancer treatment⁴⁻⁶. This thesis studies aimed at two goals: to further develop analytical techniques for DNA methylation assessment, and secondly, to validate DNA methylation markers in retrospective studies using primary GI tumor tissue. The epigenetic biomarkers' value was tested for predicting disease outcome parameters that are of specific use in GI cancer treatment.

Section 1: Technical Advances

In **chapter one** a method is presented that helps to control methylation-specific PCR (MSP) analysis of DNA samples. As a basic principle in scientific experiments, each performed assay needs to be positively and negatively controlled to allow proper, undisputed interpretation of a tested sample's result. To obtain positive controls in MSP assays means that in each experiment a sample should be amplified which contains DNA verified to have the target sequence fully methylated. Such DNA can be obtained by incubating whole genome human DNA with SssI (*Spiroplasma* sp. Strain MQ1) methyltransferase in the presence of S-adenosylmethionine (SAM) as a methyl-group donor⁷. To obtain negative controls for MSP experiments verified to have the target sequence unmethylated is very time-consuming and sometimes impossible. Mostly, DNA from peripheral blood lymphocytes is used in which usually genes of interest in cancer research show to be unmethylated. However, some genes are methylated in the healthy human genome. The first section describes a protocol to synthesize large quantities of completely unmethylated genomic DNA that can be used as a negative control in any methylation experiment, a so-called universal unmethylated control. The method is

based on that phi-29 DNA polymerase, an enzyme used for whole genome amplification does not transfer methylation patterns to its synthesized copies^{8,9}. After several rounds of amplification the amount of possibly methylated start material DNA becomes undetectable for PCR and completely unmethylated DNA is obtained. This method is cheap, useful and can be performed in any standard equipped laboratory.

In **chapter two**, a protocol for DNA preparation for methylation analysis is presented that is well suitable for tissue resources such as formalin fixed, paraffin-embedded (FFPE) tissue blocks derived from pathology department's archives. Although suboptimal for DNA evaluation, this specimen resource is highly valuable for initial retrospective assessment of biomarkers as these tissue blocks are abundantly available and long term follow up data of the patient's disease outcomes can be retrieved. Also, hematoxylin-eosin stained sections cut from FFPE tissue blocks provide excellent assessment of tumor tissue histopathology and is the golden standard. Some basic limitations have to be taken into account when working with DNA derived from FFPE tissue compared to more optimal DNA resources, i.e. from fresh frozen tissue. The formalin incubation the tumor tissue before fixation in paraffin causes DNA cleavage which results into a reduced average DNA strand length that hampers PCR amplification depending on the chosen amplicon length. Protocols to purify DNA for PCR preparation target to lose contaminating, PCR inhibiting proteins and salts. However, during purification there is much collateral loss of sample DNA. The further needed bisulfite modification (SBM) treatment again reduces and degrades the amount of sample DNA as it is incubated at 60 degrees for three hours at a pH of 5¹⁰. After SBM, several steps are needed to purify the DNA from the sodium bisulfite salts that will interfere with reaction buffers and hamper Taq-polymerase activity. **chapter two** describes the on-slide SBM protocol that was designed to reduce the number of steps needed to work up DNA for methylation status assessment. The protocol was adapted from a study by Nuovo et al¹¹. After deparaffinization, the tissue section on a glass slide is directly incubated in bisulfite solution with the DNA remaining *in-situ*, in the cell's nucleus (see **figure 1**). Our study at first compared this method with the classic used methods for bisulfite modification and shows that on-slide SBM converts DNA as efficiently as the standard protocols while harvesting significantly higher amounts of DNA. The on-slide modification dramatically reduces DNA loss as it eliminates

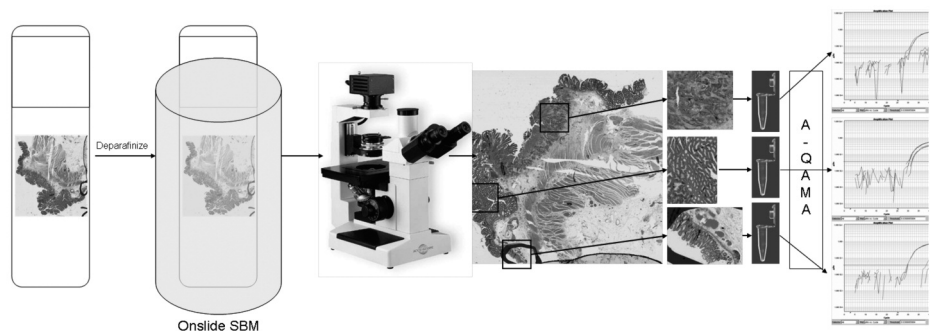


Figure 1: Schematic representation of the on-slide SBM procedure

purification steps as the DNA remains *in-situ*. Subsequently, on-slide SBM enables a high MSP success rate of tissue areas as small as 1-2 square millimeters of 7 μ m tissue sections. Other studies ensure a sufficient yield of modified sample DNA for PCR by increasing the amount of start sample. Sometimes this is achieved by putting in several whole paraffin tissue sections or lysis of complete fresh tumor tissue cubes. These methods compromise on accuracy as input DNA is not controlled for actual tumor cell content.

In the diagnostic work-up of the pathologist, usually the invasive margins of a tumor are assessed for T-stage and often in these transitional areas cells of the precursor lesion can be identified and also normal mucosa. An application of on-slide SBM is that DNA methylation status can be compared between malignant and adjacent pre-malignant and normal tissue within the same tissue section with paired analysis statistics. This is important as in other studies comparisons are often made between non-paired groups of cancer and adenoma specimens. The potential to develop into an invasive lesion is unsure when using adenoma tissue from a polyp harvested by colonoscopy. On-slide SBM therefore ensures that the adenoma represents a precursor lesion that has a proven progression into invasive carcinoma. To exemplify application, it was demonstrated in colorectal cancer that promoter region hypermethylation of the important tumor-related genes *RASSF1a*^{12,13} and *p16(INK4a)*¹⁴⁻¹⁶ occurs in the cancer stage and not in the adenoma stage. When applied in breast cancer i.e. ductal carcinoma in-situ (DCIS) areas can be compared with invasive ductal carcinoma, in cervical cancer i.e. cervical intraepithelial neoplasia (CIN) can be compared with squamous cell carcinoma. On-slide SBM can increase our knowledge of DNA methylation differences between cancers and their precursor lesions with optimal tissue histopathologic control. It may help to elucidate the changes that are specific for the transition between benign adenoma to invasive carcinoma which is the key step in malignant tumor formation.

For DNA methylation analysis our group uses capillary array electrophoresis MSP (CAE-MSP) as a standard technique for methylation analysis. CAE-MSP has several advantages over the classic MSP assay that uses agarose gel electrophoresis for PCR product detection. Gel electrophoresis is operator dependent and often requires nested PCR to obtain detectable bands which may induce non-specific amplification results. In CAE-MSP, a single round of PCR is performed with color-labeled, automatically detectable oligonucleotides with different colors for the methylation specific and unmethylation specific reaction. This enables sensitive amplicon detection in a high through-put capillary automated detection system using 96-well plates followed by automated software fragment analysis. The procedure also allows semi-quantitative interpretation of the data and confirms with each sample the predicted amplicon size length with high precision. As mentioned, CAE-MSP has excellent qualities, however, it is not a fully quantitative assay. In **chapter three**, absolute quantitative assessment of methylated alleles (AQAMA) is introduced for accurate assessment of a sample's DNA methylation level instead of methylation status. The AQAMA PCR principle was derived from QAMA described by Zegschnik et al. and is based on real-time PCR techniques. In AQAMA, two fluorescent minor-groove binding (MGB) probes, one methylation-specific and one unmethylation-specific are used to detect the amount of methylated and unmethylated alleles present in sample DNA. Important features of AQAMA that enhance reliable quantification are: 1. The use of minor groove binding (MGB) probes ensures the required high specificity to discrimi-

nate between CT (unmethylated) and CG (methylated) SBM modified sequences¹⁷⁻¹⁹. 2. Quantification is performed in a single PCR reaction while other methods use separate methylation specific and unmethylation specific reactions with different reaction kinetics. 3. The use of standard curves of dilution series of cDNA synthesized in E.Coli vectors enables absolute copy number assessment of methylated and unmethylated alleles with highly specific positive and negative control of the quantification. 4. The assay can be performed in a 384 wells system that further reduces inter-assay variability when dealing with large sample sizes as our studies intend. In **chapter three** the quantitative accuracy and linearity of AQAMA is demonstrated and its application by combining AQAMA with on-slide SBM. Tissue areas with different histopathology could be quantitatively compared for differences in methylation level instead of dichotomous comparison of methylated versus unmethylated. As an example significant differences in level of MINT locus methylation (a known specific marker for colorectal cancer) were detected between adenoma and cancer colorectal tissue. AQAMA combined with on-slide SBM can provide further quantitative detail of epigenetic events during colorectal or other solid tumor formation.

The onslide-SBM technique majorly improves and simplifies DNA preparation for DNA methylation analysis. As cells are harvested by needle scratch under a light microscope, it is inevitable that the finally analyzed sample contains some normal cells such as tumor infiltrating lymphocytes or stromal cells. To further reduce non-tumor cells sample content and to strive for analyzed DNA to be derived from 100% tumor cells, on-cap SBM was developed as described in **chapter four**. The method integrates in-situ SBM with the laser capture microdissection platform²⁰⁻²². The cells stick to a cap that can be lifted from the section and afterwards the cells can be treated, *in-situ*, with sodium bisulfite before cell digestion and continued DNA isolation. We combined on-cap SBM with the AQAMA assay to measure global hypomethylation of colorectal cancers by targeting long interspersed nucleotide element one (LINE-1), a DNA repeat that represents about 15% of the human genome. On-cap SBM followed by AQAMA of LINE-1 allowed as a first to demonstrate stepwise reduction of global hypomethylation in colorectal cancer with advanced disease stage. We also showed necessity of employing target cell selection techniques, such as laser capture microdissection, when analyzing LINE-1 in colorectal cancer.

Section 2: Clinical application

These thesis projects were initiated because of an interest to test clinical utility of epigenetic biomarkers using patient tissue of clinical databases from trials coordinated by the Leiden University Medical Center Surgical Department. Two trials were selected: the first concerned gastric cancer, the so-called D1D2 trial, and the other concerned rectal cancer, the so-called total mesorectal excision or TME-trial. The studies were initiated by the Dutch Gastric Cancer Group and the Dutch Colorectal Cancer Group, respectively. These collaborative scientists' networks have a unique infrastructure covering almost every oncologic surgical clinic in the Netherlands enabling a nation-wide cohort inclusion of GI cancer patients. As almost every citizen in the Netherlands has a family physician, patients can easily be tracked after clinical discharge which enhances complete long-term follow-up data. The clinical results of primary endpoints of these trials have been published^{23,24} and primary tumor tissue blocks were col-

lected from the different study sites pathology departments. As a consequence, the patients of the study groups in this thesis were very well documented with prospectively collected follow-up data.

The D1D2 trial compared total or partial gastric resection with D1 lymph node dissection to surgery with more extensive D2 lymph node dissection that is widely used in Asian countries and is mostly promoted by the expert gastric cancer surgeons from Japan. Almost 1000 patients were randomized in this study providing a unique patient group with a median of 14 years of follow-up at the time of our biomarker data analysis. As discussed in this thesis introduction, the multidisciplinary treatment of gastric cancer has long been frustrated by inability to show effect of (neo)adjuvant chemotherapeutics²⁵. A landmark study by McDonald et al. in 2001 was the first to show benefit of postoperative chemoradiotherapy and again gave an impulse to put scientific effort in improving treatment of gastric cancer²⁶. In 2006, Cunningham et al. showed benefit of perioperative chemotherapy over surgery alone²⁷. These important studies have created a need for biomarkers in gastric cancer to better allocate patients to the introduced aggressive treatment regimens. *Cyclo-oxygenase-2 (COX-2)* is a well studied gene in many cancers and predominantly in GI cancer. The *COX-2* enzyme is involved in production of prostaglandine PGE₂ and to a lesser extent thromboxane, both implicated in angiogenesis, cell proliferation, cell survival, migration, invasion, and modulation of host immune cells²⁸⁻³⁰. Tumors overexpressing *COX-2* have been shown to behave more aggressively during clinical follow-up in gastric cancer as well as other cancers^{31,32}. The epigenetic regulation of *COX-2* has been described in gastric cancer³³⁻³⁵. The methylation status of the *COX-2* promoter region has not been tested to have utility as a biomarker in gastric cancer. In **chapter 5** it was demonstrated that a hypermethylated status of the *COX-2* promoter region indeed leads to underexpression of COX-2 protein and subsequently to improved disease survival. A strong aspect of this study was that the predictive value was shown in two independent clinical trial populations which reduces the risk of overfitting of the data. The predictive value was independent of the established clinical parameters and therefore may have value to further aid treatment decisions in gastric cancer. From this study's conclusion it can be derived that epigenetic silencing does not necessarily result into more aggressive disease outcome of gastric cancer. DNA methylation research generated much interest because it was novel way of shut-down of tumor suppressor genes as a cause of carcinogenesis. This has lead to a paradigm that DNA methylation is an unfavorable tumor event. Since demethylating drugs are available there is an argument that they may be used to remove those unwanted methyl-groups and make the tumor-suppressor genes function again. In **chapter 5** we demonstrate DNA methylation to have a favorable role in predicting disease outcome and removal of this methylation by demethylating drugs may therefore worsen disease outcome. The data further suggest that specific gene promoter region hypermethylation may replace gene protein expression as a biomarker. DNA methylation assays have some specific advantages (i.e. non-receiver-operated, automated) and may be used instead of assessment of protein expression by immunohistochemistry when that gene is epigenetically regulated.

The Dutch TME trial on rectal cancer was the second clinical study of interest to test DNA methylation biomarker utility. The study showed improvement of the surgical treatment of rectal cancer by reducing local recurrence rates by adding short course radiotherapy befo-

re surgery²³. An important quality of this trial was the standardization of the surgical procedure using the total mesorectal excision technique described by Heald et al³⁶. Participating surgeons underwent a technical training program and were supervised during the first five operations. Furthermore, standardization of the pathology assessment of the rectal specimens controlled for completeness of the surgical treatment. The trial included 1861 patients in less than 4 years from over 108 surgical clinics. To date, epigenetic biomarkers have not been tested in rectal cancer specifically. For the study described in **chapters 6 and 7**, we established research objectives that are of specific use for rectal cancer treatment such as prediction of local recurrence and distant recurrence in early disease. In the experiments testing AQAMA and on-slide SBM we obtained encouraging data that methylation levels may have potential to subclassify rectal cancers. The required sample size for showing prognostic value was established by sample size calculations. The four MINT loci used in the AQAMA validation studies (MINT1, 2, 12 and 31) were used as markers and expanded by three more (MINT3, 17 and 25). To test if change in MINT methylation levels was related to tumor progression we first analyzed normal, adenoma and cancer tissues (**chapter 6**). We used the assay combining on-slide-SBM and AQAMA to study this. The data showed that MINT locus methylation establishes during adenomatous transformation of normal rectal epithelium and is maintained during malignant transformation. Biomarkers in other studies often positively correlate to increasing TNM-stage meaning that only a small number of cases with early disease are positive. Such markers are therefore less likely to be of clinical use in early stage patients while this group would benefit most from better subclassification. The clearly detectable and non-normally distributed MINT methylation levels in adenomas and early cancer were therefore promising to subclassify early stage disease. Then MINT locus methylation values were measured in 314 non-irradiated patients' tissues. Unsupervised random forest clustering of the data was used to analyze whether specific methylation patterns could be discerned from the data. This unconventional statistical method was opted for because of its capacity of internal validation, eliminating the need of a test-validation study set-up. Random forest (RF) clustering does not make assumptions on data distribution which makes it suitable for analyzing the non-normally distributed methylation data. Two methylation markers (MINT3 and MINT17) were identified by RF clustering that could separate the patients into four groups. Importantly, the separation was clear, with no overlap. Most biomarkers concern gene expression data with a cut-off being the median expression value. Because gene expression data are usually normally distributed, separation by this cut-off is not clear. Interestingly, an inverse relation between MINT3 and 17 methylation was present and this specific combination was required to have predictive value for distant recurrence probability in early, node-negative disease. This predictive value was not present in node-positive disease. Disease spread early by the hematogenic route may explain this finding. There is evidence of systemic spread of colorectal cancer cells in the circulation and bone marrow even in the earliest stages³⁷⁻³⁹. This indicates that very early on, tumor cells can have the capacity to enter to bloodstream and therefore may metastasize without signs of lymphatic spread. Our assay may have identified rectal tumors with such early potential for hematogenic spread. Further studies are needed to support this.

The MINT3 locus CpG island is localized on chromosome 1p34-35 just downstream of RBBP4 (retinoblastoma-binding protein ^{445,46}), SYNC1 (encoding syncollin protein involved in cell cytoskeleton and extracellular matrix proteins), YARS (tyrosyl-tRNA synthetase, involved

in angiogenesis^{47,48}) and s100p-binding protein (s100p is known to be overexpressed in many solid tumors^{49,50}). MINT17 is localized on the long arm of chromosome 12 upstream of the Harakiri (HRK) gene which is a member of the BCL2 gene family that encodes apoptosis regulatory proteins and its expression is a known target of regulation by promoter region methylation⁵¹. A functional relation between MINT3 and 17 and the mentioned genes or their expressed proteins has not been established but is an interesting subject of future studies.

5-Fluoro-uracil (5FU) -based adjuvant treatment for rectal cancer is given standard in the United States and Asia for transmurals (T3) and/or node-positive rectal cancer^{40,41} and the SCRIPT trial (www.dccg.nl) currently investigates adjuvant treatment with capecitabine (an oral form of 5FU) for this patient group in the Netherlands. The clinical utility of predicting distant recurrence in early stage rectal cancer patients becomes now important since clinicians may be hesitant to consider node-negative patients for adjuvant therapy as it is known that only 15-20 percent of these patients will show distant recurrence. Together, our results were shown in a large trial-derived patient group that was controlled for surgical and pathological quality and that was established by sample size calculations. We used preselected markers with shown potential for subclassification and obtained clearly distinct subgroups in an unbiased manner by using unsupervised random forest clustering. The predictive value in early disease patients may have utility in targeting systemic treatment after surgery due to identification of high risk on distant disease spread despite the early detection.

Another important clinical issue in the rectal cancer specifically is the risk of local recurrence due to the close anatomical relation of the rectum to the pelvis. Rectal cancer patients are therefore additionally treated with radiotherapy pre- and/or intraoperatively to improve local control. Radiation therapy in addition to surgery comes with specific long term morbidity such as more faecal incontinence, sexual dysfunction and a general reduced quality of life⁴²⁻⁴⁴. Local recurrence rates after adequate TME surgery alone are around 10% meaning that 90% will be overtreated when all rectal cancer patients undergo short-course preoperative radiotherapy. Targeting radiation treatment to those patients who are likely to locally recur could therefore be of benefit. In **chapter 7** the predictive value of MINT3 and MINT17 epigenetic biomarkers for local recurrence was evaluated. The specific combination of hypermethylation of MINT3 and decreased methylation of MINT17 was tested to be predictive for this purpose. This patient group which was at high risk for distant recurrence had significantly lower risk for local recurrence. In chapters 6 and 7 the identification of an epigenetically distinct group of rectal cancer patients who early on in their disease formation have increased potential metastasize systemically without local spread is reported. These patients may benefit more from systemic chemotherapy and less from preoperative radiation. It is important to notice that since MINT3 and MINT17 primary tumor tissue methylation levels as biomarkers can be assessed preoperatively on tumor biopsy material so it can be considered in the preoperative multidisciplinary decision making process.

In **chapter 8** the relation between quantitative MINT locus methylation and microsatellite instability (MSI) is analyzed. MSI+ colorectal cancers are a firmly established genetically distinct subgroup of sporadic colorectal cancers with mismatch repair system defectiveness and clear guidelines exist for assays to identify MSI+ cases. Sporadic MSI occurs in approximately 15% of colorectal cancers and 50% of right-sided colon cancers. Studies

have repeatedly shown a relation between DNA methylation and MSI. It is now accepted that sporadic MSI occurs through epigenetic silencing by DNA methylation of the important mismatch repair protein *hMLH1*. This is observed in tumors with genome-wide, global increased methylation at multiple CpG islands⁵². Still many issues remain unsolved. For instance at what stage of tumor formation (normal, adenoma or cancer) the relation between globally increased methylation, methylation of *hMLH1* and MSI is initiated. Our assay of on-slide-SBM combined with AQAMA is suitable to answer this specific question as paired analysis of quantitative methylation and genetic MSI analysis can be performed in different stages of CRC development. The results showed increased methylation of MINT loci and *hMLH1* in adenomatous precursors of MSI+ CRCs which showed to be MSI+ in only 50% of the cases. The data indicate global hypermethylation to precede MSI at the adenoma stage of tumor formation. Inconsistent results are published on whether MSI+ CRCs have significant better disease survival. *hMLH1* methylation is causative of sporadic MSI and this methylation is part of a global, genome-wide increased methylation of which, according to our results, MINT loci are representative. Our results show that level of MINT locus methylation has predictive value for disease outcome independent of MSI status. This result is confirmed by other groups⁵³⁻⁵⁵. The assumed improved outcome of MSI+ tumors may therefore be explained by increased MINT locus methylation levels. How high MINT locus methylation levels improve disease survival or how low MINT locus methylation enhance distant metastasis is a subject for future studies.

There are many studies that have suggested colorectal cancers with heavy methylation at CpG islands to form a clearly distinguishable subgroup of colorectal cancers, even a phenotype, the CpG island methylator phenotype (CIMP). Clinically, CIMP+ tumor features overlap with those of MSI+ tumors which are: mucinous aspect, proximal location in the large bowel and poor differentiation⁵⁵. Genetically, there is a close association with BRAF V600E mutations and KRAS wild type^{52,56}. How to define CIMP+ cases is not agreed upon as is in MSI. Different groups use different markers and different assay techniques (often non-quantitative⁵³). The frequently used quantitative MethyLight technique⁵⁶ sometimes does not measure unmethylated allele presence and normalizes the methylated allele number to a total allele count which may lead to underestimation of the degree of methylation since DNA methylation is known to be heterogeneous. Also, the quantitative data are dichotomized by a cut-off into methylated or unmethylated and the actual methylation level value is never used in clinical correlation studies. The cases showing hypermethylation in **chapter 8** likely overlap with CIMP+ patients. Our study's objective, however, was not to test whether CIMP exists, which has been established. We intended to study the quantitative relation between methylation of MINT loci and MSI+ in normal, premalignant and malignant colorectal tissue.

Concluding Remarks and Future Perspectives

Techniques

In section one of this thesis, on-slide SBM, AQAMA and on-cap SBM are presented as techniques for detailed quantitative epigenetic assessment of cancer specimens. These assays are relatively simple to perform. On-slide SBM requires no more equipment than needed

for standard immunohistochemical staining protocols. AQAMA requires a single PCR reaction on an automated real-time PCR platform. We showed its quantitative quality, its application and its potential clinical value. When interpreting results from other studies that evaluate epigenetic biomarkers consideration should be given to the techniques used. Quantitative PCR analysis of primary tumor tissue has no role in GI cancer diagnostics yet. The results of this thesis' studies show promising prospects for clinical use of AQAMA in GI cancer diagnostics. The quantitative detail of DNA methylation is yet to be further tested in other GI malignancies and solid tumors for clinical utility.

The on-slide or on-cap technique combined with AQAMA has potential to elucidate the relation of epigenetic and genetic events in the carcinogenic process of any solid tumor. This thesis' studies utilized the techniques in colorectal cancer, although the technique can compare histologically different tissue areas within a single tissue section of any type of solid tumor. The approach enabled to clarify that the relation between microsatellite instability and DNA methylation is established in the earliest stages in the oncogenic pathway of colorectal cancer. The epigenetic aspects of the histopathologically important steps in tumor formation of premalignant transformation of normal cells and invasive transformation of premalignant cells can be better studied with on-slide SBM. Applicable to any type of cancer, the opportunities of new study subjects are numerous and likely to yield interesting results.

Biomarkers

Epigenetics, like DNA methylation form a new category of molecular targets for biomarker opportunities. This thesis' studies aimed at testing DNA methylation to have utility in clinically relevant subclassification of GI cancers. The data demonstrated that specific epigenetic biomarkers in primary tumor tissue of gastric, colon and rectal cancer (the most common forms of GI cancer) can identify clearly separable subgroups of GI cancer patients and to have potential aid in making preoperative treatment decisions guiding the multidisciplinary treatment. To date, the TNM staging system is the best available tool to base treatment decisions upon and will continue to do so in the future. We show that quantitative epigenetic biomarkers can give valuable additional information in assessment of primary tumor properties for stratification of disease aggressiveness.

COX-2 methylation status was identified in this thesis' studies in gastric cancer to have prognostic value independent from TNM-stage. Only recently, effective (neo)adjuvant treatment regimens are being administered in gastric cancer. Further clinical studies have to point out what subgroups of gastric cancer patients will benefit most by these aggressive treatment regimens and biomarkers, like *COX-2* methylation analysis, will likely be of additional value. Confirmative studies are needed to test *COX-2* prognostic value in node-negative disease specifically. These analyses should be performed with a fully quantitative assay as our studies were performed with semi-quantitative techniques.

In rectal cancer we show that quantitative assessment of two MINT loci in primary tumor tissue can be of aid in specific clinical problems. Preoperative treatment planning is becoming more and more important since neoadjuvant strategies have been shown to be effective. MINT locus methylation assessed by AQAMA can help select patients for preoperative radiation therapy and similar as in colon cancer can help identify early stage, high-risk disease that may benefit from systemic treatment reducing distant metastasis. The fin-

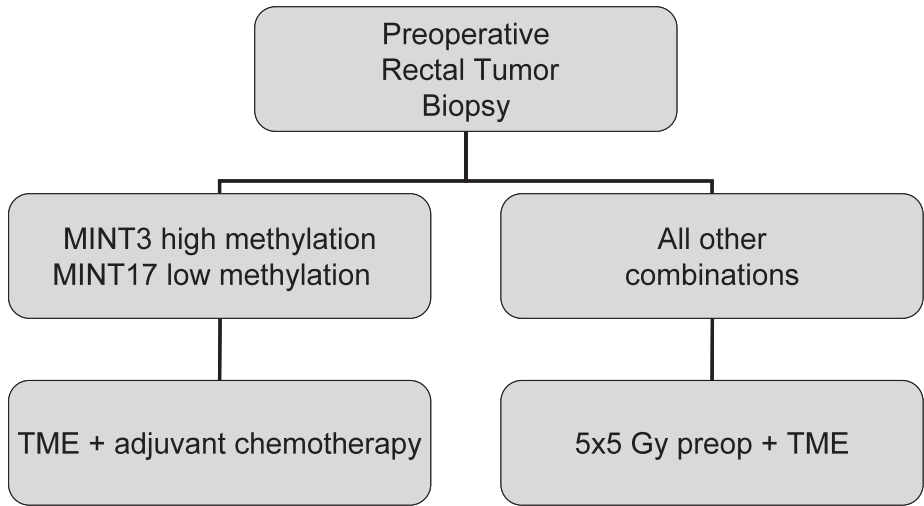


Figure 2: Suggested trial randomization schedule using *MINT3* and *MINT17* as biomarkers in non-locally advanced rectal cancers.

dings need to be validated in an independent prospectively collected patient group. If confirmed, the results may be used to design clinical trials that stratify non-locally advanced patients into high-risk local / low-risk distant recurrence or low-risk local / high-risk distant recurrence and tailor treatment according (see figure 1). In the future rectal cancer should be evaluated in biomarker studies as a separate group of large bowel cancers because it has specific treatment and specific clinical aspects to be taken into consideration.

In colon cancer, there is much discussion how to improve the selection of high-risk node negative patients for adjuvant chemotherapy. In node negative disease, primary tumor properties are becoming of clinical importance. The pathology report now additionally describes whether a tumor has signs of perineural/vascular/lymphatic invasion, however, these assessments are not routinely and systematically performed. Our studies show that MINT locus methylation is especially suitable in node-negative disease when it is detected in early disease, even at the adenomatous stage. These epigenetic biomarkers may therefore be of specific help in identifying high-risk, node-negative colon patients. Validation studies in independent, prospectively collected patient groups are needed to further investigate this.

Finally, this thesis is an example that shows the worth of close collaboration between surgical oncologists and basic scientists and demonstrates that translational medicine is of great value in cancer research to test clinical application of novel molecular targets and techniques and subsequently bring the so-called “bench” and “bedside” closer to each other.

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English Summary

Inclusion as well as exclusion

This thesis reports on colon, rectal and gastric adenocarcinoma which are the most abundant malignant tumors of the digestive system. They form a large health burden on society and this burden will only grow the coming decades with the increasing life expectancy of humans. The only chance for cure of these malignancies in most cases is radical open/laparoscopic abdominal surgery which comes with morbidity and mortality, especially in older patients. Over the last years, treatment strategies have developed towards more and more aggressive to improve survival rates. For instance, more extensive surgical resection of surrounding structures and nodal tissue or addition of systemic and local disease eradicating chemotherapeutic and radiotherapeutic modalities. We know from randomized trial data that addition of radiotherapy and/or chemotherapy before (neoadjuvant) or after (adjuvant) surgery improves the outcomes of all rectal and gastric cancers and advanced stage colon cancers. The added morbidity of these treatments affects many patients and is higher in the older patient. Neoadjuvant therapy also increases the risks of the surgical procedure and reduces the chance of a full recovery.

The clinical data demonstrate that a considerable percentage of patients will be cured with surgery alone and therefore the additional therapy is an overshoot in many patients. A better estimation of a tumor's aggressiveness beyond the current standard staging methods is an important subject to improve treatment allocation of patients. The potential benefits of improved tumor risk assessment in addition to nodal status are two-fold. First, it may allow to include more early stage patients at high risk of disease recurrence that would benefit from aggressive therapy. Secondly, it appoints low risk, early or even advanced stage patients to exclude them from undergoing unnecessary therapy.

Since it is known that neoadjuvant regimens improve disease outcomes of rectal and gastric cancer it is important to perform risk assessment preoperatively. The current diagnostic imaging modalities (CT, MRI, PET/CT) are highly important in setting up the individual treatment plan. For instance to detect distant disease spread or assess T-stage in rectal cancers. These findings are used in tailoring treatment regimens for different disease stages. The imaging modalities are used to predict the TNM-stage as best as possible. Nodal involvement remains the most important classic risk indicator for gastric and colorectal cancer survival. To determine N-stage reliably preoperatively is still not possible by imaging techniques especially in gastric and colon cancer. Primary tumor features are an attractive form of preoperative diagnostics as a tissue diagnosis obtained through gastroscopy / colonoscopy / proctoscopy is always required. Molecular primary tumor features on the DNA level are attractive because a minimum of tissue is required and the techniques are automated, robust and highly sensitive. Furthermore, the DNA molecule is highly stable compared with RNA or protein.

Epigenetics

The completion of the human genome project was a milestone in science, however, the function of only 5-10% of the genome can be explained till now. The term completion was

therefore misleading. For a long time, the other 90-95% has been called 'junk DNA', a term introduced in 1972 by Susumu Ohno. The hidden complexity of the human genome has become more evident since other genomes of life forms which are considered less complex have been unraveled. For instance, a fruit fly (*Drosophila melanogaster*) has an estimated 27,463 encoding genes compared to 31,896 in *homo sapiens*. This relatively small difference indicates that the less understood "junk DNA" may contain important functional elements that may discriminate the human and fruit fly life form. The relatively small difference of less than 9% points out that the number of genes is of lesser importance. Activation, interaction and modulation of gene expression is crucial for development of differentiated cells and the interaction of these cell in and between organ systems. The developing field of epigenetics has been shown to play a highly important role in gene regulation. The latest definition of epigenetics is that these are heritable traits (over rounds of cell division and sometimes transgenerationally) that do not involve changes to the underlying DNA sequence. One important epigenetic, DNA regulatory element has been recognized in methyl-group placement or DNA methylation. This mechanism has many important functions: specific gene silencing, X-chromosome inactivation, cell differentiation. In cancer, DNA methylation is known to be tremendously affected even more than the DNA itself. The clinical significance of changes in DNA methylation in cancer are currently being studied.

This thesis' objective is to study the role of DNA methylation in gastric, colon and rectal cancer. More specifically to explore whether these epigenetic features can be of clinical use in making multidisciplinary treatment decisions, not only to include patients that will benefit from more aggressive therapy but also to exclude patients from unnecessary therapy.

Dutch Trials

In The Netherlands an excellent infrastructure exists for the performance of multicentre clinical studies in patients with cancer. In 1989, a collaborative network of surgical oncologists started a randomized trial on surgery for gastric cancer comparing two levels of lymph node dissection. The successful surgical enterprise led to the design of more studies and expansion of the network to multidisciplinary. Today, the Dutch Colorectal Cancer Group (DCCG) is a nationwide collaboration between medical disciplines that are relevant for the diagnosis and treatment of colorectal and gastric cancer (surgical oncology, radiotherapy, medical oncology, pathology, radiology, gastroenterology). This thesis' clinical studies were performed with primary tumor tissue derived from patients that participated in two large trials conducted by the DCCG. First, the D1D2 gastric cancer trial (described above) that enrolled over 700 patients is probably the largest prospectively collected Western population of gastric cancer patients with long term follow up data up to a median of 16 years postoperatively. Second, the TME trial for rectal cancer that compared total mesorectal excision (TME) surgery with or without radiation therapy preoperatively. This trial is one of the largest trials conducted in rectal cancer specifically in Europe that included protocols to control the surgical procedure as well as the pathologic diagnostic process. The use of patient material to test clinical value of biomarkers in prospectively collected patient's tumor material is highly important and the two trials mentioned above provide well described patient study groups for researchers to interpret the results.

Section I: Technical Improvements

The studies are divided in two sections. The first section reports on four studies that describe technical improvements for analysis of DNA methylation on paraffin embedded archival tissue which was the specimen source available from the DCCG trial patients. In general, an initial assessment of the value of biomarkers is usually performed on sections of paraffin embedded tissue from pathology archives as this is an abundant tissue source and relatively easily accessible. It further allows excellent linkage of the tissue analyzed with histopathology. A problem in studying DNA methylation is that the most widely used technique requires treatment of the DNA with degrading chemicals such as sodium bisulfite modification (SBM) which makes larger amounts of input DNA necessary. This means that multiple sections of tissue or tissue punches are needed and this compromises the detail in which DNA methylation can be studied i.e. comparison of small tissue areas of a single section of HE stained tissue. We described two techniques in this section that enable such detailed analyses and that they can even compare the DNA methylation status between small groups of cells such as from early dysplastic cells in a colonic crypt with the differentiated cells of the contiguous villus.

Chapter one reports a useful protocol for researchers that perform methylation-specific polymerase chain reaction derived techniques. A protocol is described for synthesis of completely unmethylated human genomic DNA which cannot be found in nature. This DNA can be used as a negative control to include in PCR reactions that tests the DNA methylation status of any region of interest. **Chapter two** shows that SBM of unpurified DNA while it is still in the cell's nucleus on the tissue section mounted on a glass slide has an equivalent effect to treatment of DNA that is isolated and purified before SBM. Application of this method shows that it can be used to analyze small (1-2 mm²) tissue areas from a single section of PEAT and therefore one can compare DNA methylation features of areas with different types of histopathology such as tumor precursor cells and cancer cells, which was difficult to do before.

Chapter three continues with the introduction of absolute quantitative assessment of methylated alleles (AQAMA), a real-time PCR technique for assessment of DNA methylation levels. Before AQAMA our research group used capillary array electrophoresis methylation-specific PCR (CAE-MSP) which is semi-quantitative. It was shown that application of AQAMA can be used in combination with the on-slide SBM method and levels of DNA methylation can now be compared between histopathologically different tissue areas instead of two-way outcomes, methylated or unmethylated. Our data demonstrated differences in levels of DNA methylation at methylated-in-tumor (MINT) loci in colorectal polyp cells compared to contiguous cancer cells that invade the bowel wall isolated from the same tissue section. Our findings indicate the importance of assessment of methylation level instead of methylation status.

Chapter four reports on on-cap SBM which integrates the in situ SBM technique with cell isolation by laser capture microscopy. This further enhances the details in which DNA methylation can be studied. Application of on-cap SBM was demonstrated that methylation levels measured by AQAMA could be compared of long-interspersed-nucleotide-elements (LINE1) between cells isolated from the luminal side of the tumor and cells isolated from the invasive front. The major advantage of on-cap SBM is that it minimizes the pick-up of

normal cells such as fibroblasts and lymphocytes or other stromal components of a tumor. Together these chapters demonstrate not only useful techniques but also the application of these techniques that will create new possibilities of DNA methylation analysis. On-slide SBM and AQAMA have excellent qualities for assessment of large patient groups from the DCCG trials using only a few sections of paraffin embedded primary tumor tissue blocks.

Section II: clinical application

After developing robust and high-throughput techniques for processing primary tumor tissue derived from paraffin blocks we started the analysis of the DCCG's patients' material.

Over the last years more scientific effort is put in improvement of gastric cancer treatment after MacDonald et al. in 2001 showed benefit from adjuvant treatment for the first time. The recently reported improved survival rates with the addition of pre- or postoperative radio- or chemotherapy have created a need for novel markers to further classify gastric cancer patients to better tailor these newly developed regimens. Gastric cancer is known for its infiltrative component in which enzymes expressed by the tumor cells mediating the body's reaction to the tumor may play an important role. From previous work, the expression of *cyclooxygenase-2 (COX-2)*, which is known to play a key role in the inflammatory process, has been shown to be regulated by DNA methylation of its promoter region in gastric cancer in vitro. Other studies have demonstrated that patients with tumors highly expressing *COX-2* have worse disease outcome. In **chapter 5**, the hypothesis was tested that the DNA methylation status of *COX-2* is a predictor of gastric cancer disease survival. We could show in the two independent trial patient groups both well described and with follow up data over 12 years after surgery that *COX-2* methylation is a predictor of disease recurrence and survival independent of the current prognostic factors such as lymph node involvement.

COX-2 activity in gastric cancer can be assessed by staining of the tumor tissue paraffin sections with a specific antibody. This technique called immunohistochemistry is widely used in cancer diagnostics. A major problem of IHC in gastric cancer with its known difficult histopathology is the inter-individual variance of the operator that assesses the staining intensity in individual cases. There may be derived from our study's results that the expression of genes such as *COX-2* which are regulated by DNA methylation can be assessed by automated, operator independent methylation assays.

Specific targeted drugs that inactivate the *COX-2* enzyme are widely used as analgesics in rheumatic conditions. These drugs are also being tested in cancer and there are many reported studies that show probable benefit of targeted *COX-2* inhibition in gastrointestinal cancers. Another finding of our study was that our *COX-2* DNA methylation assay does correlate to *COX-2* protein expression in vivo which has not been shown before. This indicates that the *COX-2* methylation assay can also be used to select patients that will benefit from these drugs. This is especially important since an increased risk of cardiovascular events as a side-effect of specific *COX-2* inhibitors has been reported.

In **chapters six** and **seven** the findings of two studies on the clinical application of quantitative DNA methylation analysis in patients with rectal cancer are described. Rectal cancer is a clinically distinct subtype of large bowel cancer. The most important difference

lies in the close anatomical relation due to fixation of the rectum in the small pelvis compared to the colon proximal of the sigmoid fold. This makes complete surgical resection more challenging and increases the risk of local recurrence also in early stage disease, especially in tumors that lie closer to the anus. The fixation of the rectum enables directed external beam radiotherapy whereas this would not be possible in the mobile colon. The previously mentioned DCCG trial for rectal cancer evaluated a surgical technique called total mesorectal excision (TME) with or without a short course of radiotherapy before operation. TME surgery entails rectal excision en block with the surrounding rectal fat using the mesorectal fascia posterior of the fat as an anatomical border with sparing of the sacral nerves. Previous to the introduction of TME surgery by Bill Heald in the early 1980s, blunt dissection posterior of the rectum was performed that resulted into 20-25% local recurrences. The TME trial showed major improvement of this important morbidity causing disease parameter as recurrence rates were 10% after TME surgery alone and this was further reduced to 4% with the addition of preoperative radiotherapy. Despite these important findings the overall survival of rectal cancer patients did not improve and trial data analysis after five years of follow-up showed that distant disease spread determines the survival outcome.

AQAMA assays were designed to measure DNA methylation levels at seven different MINT loci that are located on 7 different chromosomes. These MINT loci were detected in a genome wide screening as differentially methylated comparing a colorectal cancer cell line with healthy DNA. MINT loci do not express any protein and have no known other function in the human genome. The MINT loci have been tested by various research groups in gastric and colorectal cancer to be of clinical value. Initially, DNA methylation levels were measured in tumor and contiguous adenoma and normal tissue. The results showed that increases of MINT methylation levels are very early events in rectal cancer and mostly establish during adenoma formation as compared with cancerous progression of adenomas. The distribution of the measured MINT methylation levels in adenoma and cancer tissue were non-normal and this indicated that the markers have potential to group rectal cancer patients. Then over 300 rectal cancer patients from the surgery-only TME trial arm were assessed by the AQAMA assays and the methylation level values were analyzed directly by a unsupervised clustering algorithm. The grouping method recognizes patterns that are present within the data in an unbiased manner. The result was that two of the MINT markers (3 and 17) were inversely correlated and can divide the patients into clearly separate groups with almost no overlap. In **chapter six** we report on the finding that this subdivision identifies a group consisting of approximately 25% of the early stage, node-negative patients that have a significantly increased risk to develop distant recurrence and subsequently reduced cancer-specific and overall survival. In **chapter seven** we report that the same group of patients with a higher chance of developing liver metastasis have a significantly lower chance of local recurrence. The DCCG previously demonstrated using the TME trial data that the patients that received radiotherapy had more fecal incontinence, sexual dysfunction, delayed wound healing and so a reduced quality of life after surgery. The other group of approximately 75% of the rectal cancer patients was analyzed and had a significantly higher incidence of local recurrence compared with irradiated patients. This

suggests that leaving out the morbidity increasing preoperative radiation can be done without disadvantaging these patients. The clustering method using only MINT3 and MINT17 was simplified using cut-offs and validated in an independent group of rectal cancer patients in order to provide an algorithm to be used by other research groups. Together the two studies report on the identification of a group of rectal cancer patients that forms about 20-25% of all patients that have a higher chance of developing liver metastasis and a lower chance of developing local recurrence after curative resection. The cluster of patients was identified by unbiased unsupervised clustering and had no overlap epigenetically with the other patients. This is important because expression values of many markers have normal distributions and often the median value is chosen as a cut-off. Most sample values are around the median and therefore such a cut-offs result into poor separation. To our knowledge this is the first study that reports on quantitative epigenetic biomarkers for rectal cancer specifically with the potential to guide the multidisciplinary treatment controlling distant and local recurrence even in early stage patients.

The final **chapter 8** focuses on colorectal adenocarcinoma precursor lesions. It utilizes the techniques described in chapter two and three and shows the value of the enhanced detail that this approach enables. About half of the right-sided colon cancers have a defective DNA mismatch repair (MMR) system caused by epigenetic silencing of the *human Mut-L homologue (hMLH1)* gene. The disruption of DNA repair results into shortening of DNA repeat sequences called microsatellites and this can be detected by simple PCR techniques. MMR defective tumors are therefore called highly microsatellite instable (MSI-H). It is widely recognized that epigenetic silencing of the *hMLH1* gene is part of a genome wide increase of methylated CpG islands including the MINT loci. We hypothesized that the quantitative AQAMA assay could detect that methylation levels at MINT loci are increased in precursor lesions of MSI-H tumors before MSI has established. The results show that the AQAMA MINT assay can be used to identify adenomas that will develop into MSI-H cancers. This finding is of biological interest as it shows that important epigenetic changes initiate in the earliest phase of colorectal cancer formation with only the presence of classic adenomatous tissue with low or intermediate dysplasia. These detectable changes early on will later lead to a distinct phenotype of colorectal cancer. Early identification of patients at risk for developing MSI-H phenotype sporadic cancers by AQAMA of MINT loci may have clinical consequences. It would be interesting to examine whether other polyps collected during colonoscopy from the same patient have similar DNA methylation features which may indicate a predisposition to colorectal cancer development. The polyp recurrence probability of patients with adenomas with high MINT locus methylation could be studied and the frequency of follow-up colonoscopies may be adjusted for such patients. Our results further show that MINT loci methylation levels can discriminate normal from adenomatous tissue. The MINT markers may therefore be a part of a screening panel tested on fecal DNA to identify patients that should undergo colonoscopy. Not only MSI-H precursor lesions may be identified by MINT markers 1, 2, 12 and 31 but also MSS lesions may be identified by MINT3 methylation. Further, it is known that MSI-H cancers respond differently to common chemotherapeutics. For instance, irinotecan is suggested to be more effective than 5-FU in these tumors. With the development of better targeted and more

patient friendly drugs, prevention strategies may be indicated for patients with polyps with high MINT locus methylation. Also, there is increasing evidence that there is MSI-induced generation of novel tumor-specific carboxy-terminal frameshift peptides (FSPs) in MSI-H cancer patients. Development of FSP-based vaccination approaches is currently ongoing and patients with polyps with high MINT locus methylation may become a target group for such vaccination trials.

Future perspectives

All together this dissertation reports on improvement of molecular techniques for studying DNA methylation and describes application of the techniques to identify biomarkers with the potential to improve the treatment of gastric, colon and rectal cancer. The next step was taken to further develop the clinical use of the described biomarkers. The prognostic value of *COX-2* methylation status as a biomarker in gastric cancer was shown in a second independent, trial-derived patient group. Subsequently, a clinical trial could be suggested that allocates patients with a methylated *COX-2* promoter region to surgery alone leaving out neoadjuvant therapy. The primary study objective would be to show that patients in both trial arms have no significant differences in disease outcome.

Future studies continuing the work on MINT3 and MINT17 as biomarkers in rectal cancer would be to confirm the results of the predictive value for distant and local recurrence in an independent group of rectal cancer patients treated with and without preoperative radiotherapy. If confirmed, an interesting trial design can be proposed as follows. MINT3 and MINT17 methylation index is measured in the preoperative tumor biopsy tissue. The 20-25% of the specific risk cluster undergo TME surgery without preoperative radiotherapy and do receive postoperative chemotherapy. The other 75-80% of the patients receive 5x5Gy preoperative radiation and if node-positive or T3N0, postoperative chemotherapy can be considered. The latter consideration may be supported by the outcome of the SCRIPT trial that is currently being conducted.

Samenvatting Nederlands

Biomarkers: voor inclusie en exclusie

Dit proefschrift heeft als onderwerp maagkanker en dikke darmkanker. Dikke darmkanker kan onderverdeeld worden in het colon carcinoom (vanaf de klep van Bauhin tot en met de S-bocht) en het rectum carcinoom (endeldarmkanker). Deze maligniteiten ontaarden uit de cellen van het bekledende slijmvlies van darm en maag en zijn de meest voorkomende vormen van kanker van het spijsverteringsstelsel. Zij zorgen voor een aanzienlijk deel van de kankersterfte, niet alleen in Nederland maar ook wereldwijd. Hiernaast vormen ze een grote belasting voor de Nederlandse gezondheidszorg en met het stijgen van de levensverwachting zal dit alleen maar toenemen. De enige kans op overleving in het overgrote deel van de gevallen bestaat uit het radicaal verwijderen middels open of laparoscopische buikchirurgie. Deze operaties kennen een niet gering risico op complicaties en sterfte. Deze risico's nemen toe bij oudere patiënten. Om de overlevingskansen van maag- en darmkankerpatiënten te vergroten worden behandelstrategieën steeds agressiever. Men moet dan bijvoorbeeld denken aan het wegnemen van meer omliggende structuren en klierweefsel tijdens de operatie of het toevoegen van radiotherapie en/of chemotherapie voor en/of na de operatie. Door middel van gerandomiseerde studies is aangetoond dat voorbehandelen met chemotherapie de overleving verbetert in aanvulling op een radicale chirurgische verwijdering van maagkanker. Zo ook resulteert het voorbestralen van rectum tumoren in combinatie met het ruimer wegnemen van omgevend vet en bindweefsel tijdens de operatie in een aanzienlijke afname van een recidief tumor lokaal in het geopereerde gebied. De toegevoegde morbiditeit van deze aanvullende behandelingen treffen veel patiënten en komt wederom vaker voor bij de oudere patiënt. Voorbehandelen met chemo- en/of radiotherapie vergroot eveneens het risico op complicaties van de operatie en vermindert de kans op een volledig herstel. De data van klinische studies die chirurgie in combinatie met een aanvullende behandeling vergelijken met chirurgie alleen laten ook zien dat een aanzienlijk deel van de patiënten gebaat zouden zijn bij alleen een operatie. Dit wijst erop dat deze nabehandeling, oftewel adjuvante therapie vaak een overbehandeling is. Een betere inschatting van de agressiviteit van maag- en darmtumoren in aanvulling op de huidige stadiëringsmethoden is daarom een belangrijk onderwerp om patiënten te kunnen selecteren die ook daadwerkelijk baat hebben bij een agressievere behandeling. De huidige belangrijkste voorspeller van overleving van maag- en dikke darmtumoren is het al dan niet vinden van uitzaaiingen in de regionale lymfeklieren. De voordelen van verbeterde risico-inschatting in aanvulling op deze parameter zijn tweeledig. Aan de ene kant zouden hoog-risico, klier-negatieve patiënten geïnccludeerd kunnen worden in trials om het nut van aanvullende therapie te testen. Aan de andere kant zouden laag-risico, klier-negatieve en mogelijk ook klier-positieve patiënten geëxcludeerd kunnen worden van morbiditeit- en mortaliteit verhogende aanvullende therapie.

Het is bekend dat voorbehandelen (neoadjuvant) de uitkomst van ziekte van endeldarmkanker en maagkanker na radicale chirurgie verbetert, is het dus ook van belang om de risico-inschatting voorafgaand aan de operatie te kunnen doen. Beeldvormende technie-

ken zoals CT-, MRI- en PET/CT-scans zijn van grote waarde bij het opstellen van een individueel toegespitst behandelplan. Bijvoorbeeld voor metastaseringsonderzoek of voor het identificeren van “locally advanced, T3/4” rectum tumoren. Deze onderzoeken worden gebruikt om zo goed mogelijk het TNM-stadium in te schatten vóór de operatie. De N-stadiëring die de belangrijkste voorspeller is, kan echter niet met 100% betrouwbaarheid worden bepaald met de huidige beeldvorming en met name niet in het geval van maag- en coloncarcinomen. Moleculaire kenmerken van de primaire tumor op DNA niveau zijn een aantrekkelijk preoperatief diagnosticum omdat deze bepaald kunnen worden in tumorbiopsen verkregen met scopisch onderzoek die horen bij het standaard diagnostisch proces. DNA kenmerken zijn verder aantrekkelijk vanwege de chemische stabiliteit van het molecuul waardoor slechts een minimale hoeveelheid weefsel nodig is.

Epigenetica

De voltooiing van het “human genome project” in 2003 dat de nucleotide-volgorde van het menselijk genoom in kaart bracht was een mijlpaal in de wetenschap. Echter is de functie van slechts 5-10% van het genoom bekend. De term “voltooiing” van dit project kan daarom misleidend genoemd worden. De resterende 90-95% van het genoom is lange tijd “junk DNA” genoemd, een term geïntroduceerd in 1972 door Susumu Ohno. De verborgen complexiteit van het menselijk genoom werd nog eens benadrukt na de ontcijfering van volledige genomen van andere levensvormen die gezien worden als minder complex. Het genoom van een fruitvlieg (*Drosophila Melanogaster*) heeft bijvoorbeeld een geschat aantal van 27.463 coderende genen vergeleken met de 31.896 bij de mens (*Homo Sapiens*). Dit relatief kleine verschil wijst er op dat in het onbegrepen deel “junk DNA” belangrijke elementen verborgen moeten liggen die het verschil maken tussen een mens en een fruitvlieg. Het verschil van minder dan 9 percent laat dus zien dat het aantal genen van ondergeschikt belang is. Activering, interactie en modulatie van expressie van genen is dan ook cruciaal voor de vorming van gedifferentieerde cellen en de onderlinge communicatie tussen gedifferentieerde celstructuren binnen en tussen orgaansystemen. Het zich ontwikkelende gebied van epigenetica heeft aangetoond een zeer belangrijke rol te hebben in gen regulatie. De laatste definitie van epigenetica is dat dit overerfbare elementen zijn van cel op cel, en soms van generatie op generatie, die geen betrekking hebben op veranderingen in de onderliggende DNA volgorde. Een belangrijk DNA regulerend element is methylgroep plaatsing op cytosine-guanine dinucleotiden oftewel DNA methylering. Enkele belangrijke fenomenen kunnen inmiddels verklaard worden door DNA methylering. Bijvoorbeeld: X-chromosoom inactivatie, cel differentiatie, DNA stabilisatie en specifieke gen inactivatie bij kanker. Veranderingen in DNA methylering worden meer/vaker gezien in kankercellen in vergelijking met gezonde cellen. DNA methylering is zelfs meer afwijkend in kankercellen dan het DNA zelf. De klinische betekenis van veranderingen in DNA methylering in kanker vormen een onderwerp van veel studies.

Het doel van de studies beschreven in dit proefschrift is om de rol van DNA methylering te bestuderen in maag-, colon- en rectumcarcinomen. En meer specifiek om te exploreren of deze epigenetische elementen in de primaire tumor gebruikt kunnen worden als markers om de multidisciplinaire therapie verder te kunnen sturen. Het gaat hierbij niet

alleen om patiënten te kunnen includeren die baat zullen hebben van meer agressieve behandeling, maar het gaat er ook om patiënten te kunnen excluderen van onnodig belastende therapie.

De Nederlandse Trials

In Nederland bestaat een uitstekende infrastructuur om multicenter studies uit te voeren bij kankerpatiënten. Een samenwerkingsverband van chirurgisch oncologen startte in 1989 een gerandomiseerde trial naar chirurgie voor maagcarcinoom die twee niveaus van lymfeklierdissectie met elkaar vergeleek. Deze succesvolle trial leidde tot meer studies en multidisciplinaire uitbreiding van de samenwerking. Het resulteerde in de oprichting van de “Dutch Colorectal Cancer Group” (DCCG). Vandaag de dag is de DCCG een landelijk samenwerkingsverband tussen medische disciplines (o.a. chirurgen, radiotherapeuten, internist-oncologen, pathologen, radiologen en gastro-enterologen) en is betrokken bij de diagnose en behandeling van colorectale en maagtumoren. Dit proefschrift bevat enkele studies waarbij gebruik werd gemaakt van weefsel van primaire tumoren van patiënten die deelnamen in twee grote trials gecoördineerd door de DCCG. Ten eerste, de zogenaamde D1D2 gerandomiseerde chirurgische trial voor patiënten met maagcarcinoom (zie hierboven) met meer dan 700 geïncludeerde patiënten. Deze trial is mogelijk de grootste prospectief verzamelde Westerse populatie van patiënten met maagcarcinoom met follow-up data gemiddeld tot 14 jaar postoperatief. Ten tweede, de zogenaamde TME trial voor patiënten met rectum carcinoom die totale mesorectale excisie (TME) chirurgie met en zonder vooraf radiotherapie vergeleek. Deze trial is één van de grootste studies specifiek naar rectumcarcinoom met protocollen om de chirurgische procedure en het pathologisch diagnostisch proces te controleren. De kwaliteit van studies die gebruik maken van humaan materiaal om klinische waarde van biomarkers te evalueren staat of valt met de oorsprong van de patiëntgroep. Prospectieve verzameling, uniforme behandeling, uniforme diagnostiek en uniforme follow-up zijn van groot belang voor andere onderzoekers om de resultaten te kunnen interpreteren. De kwaliteit en omvang van de D1D2 en TME trial heeft dus in belangrijke mate bijgedragen aan de resultaten beschreven in dit proefschrift.

Sectie I: Technische verbeteringen.

Het proefschrift is verdeeld in twee secties. De eerste sectie behelst vier studies die gaan over technische verbeteringen bij de analyse van DNA methylering in weefselcoupes afkomstig van gearhiveerd, met paraffine gefixeerd tumor materiaal. Dit paraffine materiaal was de beschikbare bron van de DCCG trial patiënten. Gewoonlijk wordt voor een initiële bepaling van de waarde van biomarkers paraffine materiaal gebruikt. Simpelweg omdat vele coupes van enkele micrometers dik gesneden kunnen worden en het weefsel makkelijk toegankelijk ligt opgeslagen in de archieven van de patholoog. Verder geven paraffine coupes het voordeel van direct visualisatie van de histopathologie. Een probleem is dat de meest toegepaste techniek voor analyse van DNA methylering gebruikt maakt van langdurige incubaties met DNA degraderende chemicaliën (sodium bisulfit modificatie oftewel SBM). Daarnaast resulteren DNA isolatie- en zuiveringsmethoden in verlies van veel DNA. Veel studies beschrijven dan ook het gebruik van grote hoeveelheden startmateriaal bestaande uit meerdere gehele coupes of zelfs blokjes tumorweefsel. Dit compromit-

teert het detail van het startmateriaal en verhoogd de kans op contaminatie met gezond weefsel. In sectie I worden twee technieken beschreven die het mogelijk maken om DNA methylering te bepalen in een minimale hoeveelheid startmateriaal zoals 1-2 vierkante millimeter van een 7 micrometer dikke weefselcoupe of van enkele duizenden cellen geïsoleerd door middel van laser capture microscopie. Hiermee kan dus in potentie DNA methylering vergeleken worden tussen vroege dysplastische cellen in een crypte van het colon slijmvlies met de gezonde gedifferentieerde cellen van de aanliggende villus.

Hoofdstuk één beschrijft een nuttig protocol voor onderzoekers die methylering-specifieke polymerase chain reaction (PCR) technieken gebruiken of afgeleiden daarvan. Het protocol behelst de synthese van compleet ongemethyleerd humaan genomisch materiaal dat niet voorkomt in de natuur. Dit DNA kan gebruikt worden als een negatieve controle in PCR reacties om de methyleringsstatus te testen van iedere gewenste regio van het genoom.

Hoofdstuk twee test de behandeling van coupes gedeparaffiniseerd weefsel op glazen **direct** met sodium bisulfiet, zogenaamd on-slide sodium bisulfiet modificatie (SBM). Resultaten laten zien dat deze behandeling van het ruwe DNA, nog opgeslagen in de celkern (*in situ*), dezelfde resultaten heeft als wanneer van geïsoleerd DNA opgelost in water gebruik wordt gemaakt. Omdat de isolatie- en zuiveringstappen overgeslagen kunnen worden resulteert onslide-SBM in een veel hogere DNA opbrengst voor verdere PCR analyse. Als toepassing van deze bevinding wordt aangetoond dat DNA methylering van kleine stukjes weefsel van 1-2 vierkante millimeters van één enkele coupe paraffine materiaal met elkaar vergeleken kan worden. Als voorbeeld was in een colon tumor de promotor-regio van een belangrijk tumor-gerelateerd gen gemethyleerd in carcinoom weefsel en ongemethyleerd in het aanliggend weefsel met daarin benigne voorloper cellen. Dit soort analyses waren voorheen moeilijk uitvoerbaar en onslide-SBM maakt dit aanzienlijk eenvoudiger.

Hoofdstuk drie beschrijft een kwantitatieve PCR techniek genaamd AQAMA (absolute quantitation of methylated alleles). Onze onderzoeksgroep gebruikte hiervoor klassieke methylerings-specifieke PCR met capillaire array elektroforetische analyse van de PCR producten (CAE-MSP) die slechts semi-kwantitatief is. Als toepassing werd AQAMA gecombineerd met *in situ*, on-slide SBM. DNA methylerings-gehalten van specifieke genomische loci kunnen nu vergeleken worden met histopathologisch verschillende weefselstukjes van dezelfde paraffine coupe in plaats van met een dichotome uitkomst: gemethyleerd of ongemethyleerd. De resultaten lieten significante verschillen zien in gehalte DNA methylering van methylated-in-tumor (MINT) loci tussen colon adenomateus en carcinoom weefsel. De bevindingen illustreren het belang van bepaling van het methylerings-gehalte in plaats van methylerings-status.

Hoofdstuk vier gaat over de zogenaamde “on-cap SBM” van DNA die de eerdere *in situ* behandeling van een weefselcoupe op een glasje (on-slide) integreert met laser-capture microscopie. Met deze techniek kunnen individuele cellen of groepen cellen van een paraffine coupes geïsoleerd worden. On-cap SBM verbetert dus het detail van DNA methylering analyse van kleine stukjes weefsel van een paraffine coupe naar individuele cellen. Als toepassing werd het methyleringsgehalte van DNA repetitieve elementen (long-interspersed-nucleotide-elements oftewel LINE-1) gemeten met AQAMA en vergeleken met colon kankercellen van de lumenale zijde van een tumor en de kankercellen geïsoleerd van

het invasieve front. Het belangrijkste voordeel van on-cap SBM is dat het contaminatie met fibroblasten, lymfocyten en ander stromale componenten tot een minimum beperkt.

Bij elkaar beschrijven de studies van Sectie I niet alleen nuttige technieken maar ook hun toepassing en dat ze nieuwe vormen van DNA methylering-analyses mogelijk maken. Daarbij komt dat AQAMA gecombineerd met on-slide SBM een geschikte techniek is voor de analyse van het primaire tumor materiaal van grote patiëntengroepen van de DCCG trials.

Sectie II: Klinische toepassing

Na robuuste en “high-throughput” technieken te hebben ontwikkeld voor paraffine materiaal is er gestart met de analyse van tumor materiaal van patiënten van de D1D2 en TME trial.

De behandeling van maagkanker staat weer meer in de belangstelling omdat recent is aangetoond dat chemotherapie voorafgaand aan chirurgie de overleving verbeterd. Deze ontwikkeling schept een behoefte aan het ontwikkelen van voorspellende, preoperatief te bepalen biomarkers, om risico-inschatting van patiënten met maagcarcinoom te verbeteren. Zo kan wellicht de chemotherapie toegespitst worden op patiënten met een meer agressieve ziekte. Het maagcarcinoom is bekend om zijn infiltrerende, infectieuze component waarbij door de tumor tot expressie gebrachte enzymen, die de ontstekingsreactie van het lichaam kunnen reguleren, mogelijk een belangrijke rol spelen. Eerdere studies laten zien dat de expressie van *cyclooxygenase 2 (COX-2)*, welke een sleutelrol speelt in het inflammatoire proces, wordt gereguleerd door DNA methylering van zijn promotor regio in maagcarcinoom. Andere studies hebben laten zien dat patiënten met maagtumoren met een hoge expressie van *COX-2* een slechtere overleving hebben.

Hoofdstuk vijf beschrijft de resultaten van een studie met als hypothese dat de promotor regio DNA methylerings-status van *COX-2* in primaire tumor cellen een voorspeller is van de ziekteoverleving van maagkanker. In twee onafhankelijke trial patiënt groepen met een mediane follow up van twaalf jaar na chirurgie kon worden aangetoond dat *COX-2* methylering een voorspeller van ziekte recidief en overleving is onafhankelijk van alle bekende prognostische factoren zoals lymfeklier metastasen. *COX-2* activiteit in maagkanker kan worden bepaald met kleuring van tumorweefsel coupes met een specifiek antilichaam. Deze immunohistochemische techniek heeft als nadeel bij maagkanker (bekend om zijn moeilijke histopathologie) dat er een inter-individuele bias ontstaat bij het scoren van de mate van aankleuring onder de microscoop. De resultaten van de studie suggereren dus dat bepaling van de eiwit expressie door middel van immunohistochemie van genen zoals *COX-2*, die gereguleerd worden door DNA methylering, vervangen kan worden door een geautomatiseerde, operatoronafhankelijke PCR test. Verder bestaan er geneesmiddelen die specifiek *COX-2* remmen en deze worden gebruikt als analgetica bij reumatische aandoeningen. De toepassing van *COX-2* remmers ter voorkoming van darmpoliepen is onderzocht en laat een gunstig effect zien. Onze resultaten toonden aan dat er een correlatie bestaat tussen de *COX-2* methylerings test en de *COX-2* eiwit expressie. Mogelijke kan deze test dus gebruikt worden om patiënten te selecteren die baat hebben bij een therapie met *COX-2* remmers. Dit is van belang omdat cardiovasculaire bijwerkingen in een klein percentage van de behandelde patiënten is aangetoond.

In de **hoofdstukken zes en zeven** worden de bevindingen beschreven van twee studies naar de klinische toepassing van kwantitatieve DNA methyleringsanalyse bij patiënten met rectumcarcinoom. Rectum tumoren vormen klinisch een aparte groep van dikke darm tumoren. Het belangrijkste verschil is de nauwe anatomische relatie door de fixatie van het rectum in het kleine bekken vergeleken met het colon proximaal van de omslagplooï. Dit maakt een radicale chirurgische resectie tot een grotere uitdaging. Rectumtumoren hebben dan ook een groter risico op lokaal recidief, zelfs in vroege stadia. Dit risico neemt toe naarmate de tumoren dichterbij de anus gelokaliseerd zijn. De fixatie van het rectum in het kleine bekken maakt het toepassen van externe radiotherapie mogelijk hetgeen niet mogelijk is in het mobiele colon. De hierboven genoemde DCCG trial voor rectumcarcinoom evalueerde een chirurgische techniek genaamd Totale Mesorectale Excisie (TME) wel of niet voorafgegaan door een korte kuur radiotherapie. TME chirurgie is een rectumextirpatie met en-bloc resectie van het omliggende rectale vet welke gebruikt maakt van de mesorectale posterieure fascie als een anatomische scheidingsvlak met sparen van de sacrale zenuwen. Voor de introductie van TME chirurgie door Bill Heald in de vroege jaren tachtig van de vorige eeuw was het stomp vrijprepareren posterieur van het rectum (vaak met de hand) gebruikelijk. Deze techniek resulteerde in 20-25% lokaal recidief. De TME trial liet echter een forse afname zien van deze belangrijke morbiditeit veroorzakende parameter van 10% lokaal recidief na TME chirurgie alleen. En nog een significante verdere reductie naar 4% met de toevoeging van preoperatieve radiotherapie. Ondanks deze belangrijke verbetering verschilde de algehele overleving 6 jaar na de operatie niet tussen de twee groepen en lieten deze analyses zien dat overleving wordt bepaald door het optreden van uitzaaiingen op afstand.

AQAMA tests werden ontwikkeld om het methyleringsgehalte te bepalen van zeven verschillende MINT loci in DNA gelokaliseerd op zeven verschillende chromosomen. Deze MINT loci werden ontdekt als gehypermethyleerd in een genoom-wijde screen van een bekende coloncarcinoom cellijn vergeleken met gezond DNA. MINT loci zijn zogenaamde CpG eilanden van niet coderende gebieden DNA en hadden tot zover bekend geen functie. MINT loci zijn echter door diverse onderzoeksgroepen bestudeerd in maag- en darmkanker en bleken wel van klinische waarde te zijn. In onze studie werd allereerst het DNA methyleringsgehalte van de MINT loci onderzocht in tumorweefsel en aangrenzend adenomateus en normaal weefsel. Dit liet zien dat toename van MINT methylering vroeg optreedt in rectale carcinogenese, met de grootste toename tijdens adenomateuze verandering van normaal weefsel in vergelijking met carcinomateuze verandering van adenomen. De distributie van de gemeten MINT methyleringsgehalten in tumoren en adenomen was niet normaal en dit wees erop dat de data gebruikt konden worden voor clusteranalyse. Vervolgens werd in primair tumorweefsel van meer dan 300 patiënten van de chirurgie arm van de TME trial methyleringsgehalte bepaald van de zeven MINT loci met AQAMA. Deze data werden geanalyseerd met een ongesuperviseerde clustermethode om patronen te identificeren zonder bias. De resultaten lieten zien dat MINT loci 3 en 17 een sterke negatieve correlatie met elkaar hadden en op basis van deze twee biomarkers konden de patiënten ingedeeld worden in duidelijk te onderscheiden groepen zonder overlapping.

Hoofdstuk zes rapporteert de bevinding dat deze onderverdeling een groep van vroeg stadium rectum carcinoom patiënten identificeert van ongeveer 25% van het totaal. Deze patiënten hadden significant meer risico om levermetastasen te ontwikkelen en hadden een kortere overlevingstijd na curatieve chirurgie.

In **hoofdstuk zeven** wordt beschreven dat dezelfde groep patiënten met een hogere kans op uitzaaiingen op afstand een significant lagere kans hadden op lokaal recidief. De DCCG heeft in publicaties met TME trial data laten zien dat patiënten die vooraf bestraald werden meer kans hadden op fecale incontinentie, seksuele dysfunctie, vertraagde wondgenezing en verminderd kwaliteit van leven na chirurgie. Het percentage lokaal recidief van de overige 75% van de patiënten was significant hoger vergeleken met bestraalde patiënten uit de TME trial terwijl van de andere 25% dit iets lager was (niet significant). Dit suggereert dat een kwart van de patiënten met rectumcarcinoom, geïdentificeerd door middel van AQAMA van MINT 3 en 17, veilig geëxcludeerd kunnen worden van morbiditeit verhogende preoperatieve bestraling.

Bij elkaar beschrijven **hoofdstuk zes** en **zeven** de identificatie van een groep bestaande uit ongeveer een kwart van de patiënten met rectumcarcinoom welke een significant hoger risico heeft op het ontwikkelen van afstandsrecidief en een significant lager risico op het ontwikkelen van lokaal recidief. In veel biomarker studies worden groepen gevormd door middel van een afkappunt bij een mediane waarde van een normaal verdeling. Omdat de meeste waarden liggen rondom de mediaan leiden dit soort afkappmethoden tot een slechte scheiding van de groepen. Een belangrijk gegeven is dat de groep in onze studie werd herkend door een onge-superviseerde clustermethode zonder bias en dat de clusters geen overlap met elkaar hebben. Onze studie is de eerste die laat zien dat kwantitatieve epigenetische biomarkers specifiek in rectumcarcinomen de multidisciplinaire behandeling kunnen sturen in de richting van controle op afstands- of lokaal recidief, zelfs bij tumoren in een vroeg stadium.

Het laatste **hoofdstuk acht** richt zich op voorloper laesies zoals poliepen en adenomen van dikke darmkanker. Er wordt gebruik gemaakt van de technieken beschreven in hoofdstuk twee en drie (onslide SBM en AQAMA) en laat de meerwaarde zien van meer gedetailleerde analyses. Ongeveer de helft van de rechtzijdige coloncarcinomen hebben een aangeast DNA “mismatch repair (MMR)” systeem dat wordt veroorzaakt door epigenetische afschakeling van het *human mut-l-homologue (hMLH1)* MMR gen door DNA methylering van de promoter regio. Het falende MMR systeem resulteert in verkorting van zogenaamde microsatelliet regio's hetgeen eenvoudig bepaald kan worden door middel van PCR techniek. MMR aangedane tumoren worden ook wel microsatelliet instabiel genoemd. Het is aangetoond dat de epigenetische afschakeling van *hMLH1* een onderdeel is van een genoom wijd toegenomen methylering van promoter regio's en ook van MINT loci. We onderzochten in **hoofdstuk acht** de hypothese dat methyleringsgehalte van MINT loci verhoogd is in voorloper laesies van microsatelliet instabiele tumoren. De resultaten laten zien dat AQAMA van specifieke MINT loci adenomen kunnen identificeren die zich zullen ontwikkelen tot een microsatelliet instabiel colon carcinoom. Deze bevinding is biologisch relevant omdat het laat zien dat epigenetische veranderingen ontstaan in de vroegste fase van colorectale tumorvorming waarin histopathologisch slechts polypeus weefsel met laag tot gemiddelde dysplasie is te zien. Deze veranderingen leiden veel later tot een apart fenotype darmkanker.

Een mogelijke klinische toepassing is dat sommige MINT markers gebruikt kunnen worden als onderdeel van een panel om fecaal DNA te screenen om patiënten te selecteren voor colonoscopie. Verder is bekend dat microsatelliet instabiele tumoren anders reageren op chemotherapie. Het veel gebruikte 5-fluorouracil is bijvoorbeeld minder effectief in deze tumoren, echter reageren ze beter op bijvoorbeeld irinotecan. Preventieve specifieke behandelstrategieën zijn dan ook denkbaar bij patiënten bij wie met colonoscopie veel poliepen met hoge MINT locus DNA methylering worden gevonden. Verder is bekend dat bij patiënten met microsatelliet instabiele tumoren zogenaamde frameshift peptiden detecteerbaar zijn. Deze peptiden kunnen middels antilichamen een immuunreactie van het lichaam tegen de tumorcellen opwekken. Poliepvormende patiënten met hoge DNA MINT locus methylering in hun adenomen kunnen dan ook mogelijk baat hebben van vaccinatie studies.

De toekomst

Dit proefschrift beschrijft verbeteringen van moleculaire technieken voor bestudering van DNA methylering en beschrijft toepassingen van deze technieken om biomarkers te identificeren die potentieel de behandeling verbeteren van patiënten met maag-, colon- en rectumcarcinoom. Bij de studie naar *COX-2* methyleringsstatus als prognostische marker bij maagcarcinoom werd ook de volgende stap gezet, namelijk validatie in een tweede onafhankelijke groep patiënten eveneens afkomstig van een gerandomiseerde klinische studie. Het is nu mogelijk een klinische trial te ontwerpen waarbij als volgt kan worden gerandomiseerd: patiënten waarvan het preoperatief tumor biot een gemethyleerd *COX-2* gen laat zien worden direct geopereerd zonder perioperatieve therapie terwijl de patiënten met een ongemethyleerd *COX-2* gen neoadjuvant behandeld zullen worden. Aan de hand van de resultaten van de CRITICS trial kan dan worden besloten om *COX-2* ongemethyleerde en/of klier positieve tumoren dan adjuvant te behandelen met chemo-radiotherapie. De primaire uitkomstmaat van de studie zou moeten zijn dat klier-negatieve, *COX-2* gemethyleerde tumoren behandeld met chirurgie alleen een niet significant verschillende overleving hebben vergeleken met de wel aanvullend behandelde patiënten.

Toekomstige studies die verder gaan met MINT3 en MINT17 als biomarkers in rectumcarcinoom dienen zich allereerst te richten op de validatie in een onafhankelijke groep patiënten behandeld met en zonder preoperatieve radiotherapie. Indien de voorspellende waarde voor afstands- en lokaal recidief kan worden bevestigd dan kan de volgende trial worden ontworpen; Allereerst wordt de MINT3 and MINT17 methylerings index bepaald in weefsel van het preoperatieve tumorbiot. Indien er sprake is van een non-locally advanced tumor zoals bepaald per MRI onderzoek ondergaat ongeveer 25 procent van de patiënten van het specifiek risicocluster TME chirurgie zonder voorbestraling maar wel met postoperatieve systemische chemotherapie. Alle locally advanced tumoren ondergaan de meer uitgebreide neoadjuvante behandeling en afhankelijk van de MINT3 en MINT17 methyleringsstatus al dan niet adjuvante therapie. De andere 75% van de non-locally advanced patiënten ondergaan 5x5Gy preoperatieve radiotherapie gevolgd door TME chirurgie. De uitkomsten van de SCRIPT trial kunnen verder bepalen of patiënten met lymfekliermetastasen of zonder lymfekliermetastasen waarvan de tumor door de hele darmwand groeit nog verder baat zullen hebben van nabehandeling met het goed verdraagbare oraal in te nemen Xeloda (5-fluorouracil).

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During my translational studies I have had the pleasure of working with a great number and variety of people. This thesis' work would not have been possible without the help of the clinicians and molecular scientists of the Leiden surgical oncology group and of the John Wayne Cancer Institute in Santa Monica. I would like to thank all the people standing at the laboratory benches and patient bedsides and some people in particular:

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Curriculum Vitae

The author of this thesis was born June 1st 1979 in Baexem in the South of the Netherlands. After graduation from the Saint Ursula high school (gymnasium beta) in Horn he went to the Technical University of Delft to study architecture in 1997. One year later he was admitted to the Leiden University medical school. In his third year he undertook his first science projects at the surgical department's oncology laboratory headed by professor Cock van de Velde and Peter Kuppen, PhD. This was followed by a doctoral degree graduation project on surgery for hyperparathyroidism supervised by professor Job Kievit.

He was given the opportunity to visit the John Wayne Cancer Institute's department of gastrointestinal surgery in Santa Monica, California (head Dr. Anton Bilchik) in 2002 after obtaining his doctoral degree. This led to a research fellowship at JWCI's department of molecular oncology, head Dr. Dave Hoon in collaboration with the LUMC's surgical department for which he interrupted his medical studies. In this period the studies for this thesis were conducted.

He resumed medical school in 2006 and obtained his MD in 2008 (cum laude, with honors). After working for one year in the Haga hospitals in the Hague he recently joined the Orbis Medical Center's surgical department in Sittard.

List of Publications

- **Identification of a Quantitative MINT Locus Methylation Profile Predicting Local Regional Recurrence of Rectal Cancer**
de Maat MF, van de Velde CJ, Benard A, Putter H, van Krieken J, Meershoek-Klein Kranenbarg E, Tollenaar RA, Hoon DS
Accepted for publication by Clinical Cancer Research
- **CpG island methylator phenotype predicts progression of malignant melanoma.**
Tanemura A, Terando AM, Sim MS, van Hoesel AQ, de Maat MF, Morton DL, Hoon DS.
Clin Cancer Res. 2009 Mar 1;15(5):1801-7. Epub 2009 Feb 17.
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- **Assessment of methylation events during colorectal tumor progression by absolute quantitative analysis of methylated alleles.**
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- **Methylation of p16 and Ras association domain family protein 1a during colorectal malignant transformation.**
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- **Development of Sporadic Microsatellite Instability in Colorectal Tumors Requires Hypermethylation at Methylated-IN-Tumor Loci in Adenoma**
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Submitted for publication

- **LINE-1 methylation analysis in colorectal cancer shows progression of hypomethylation at early stage**
Sunami E, de Maat MF, Vu A, Turner RR, Hoon DS
Submitted for publication
- **Mutant Methyltetrahydrofolate-reductase (MTHFR) Polymorphisms Results in Improved Gastric Cancer Disease Outcome**
de Maat MF, Hiramatsu S, Benard A, Meijer G, Putter H, Tollenaar RA, van de Velde CJ, Hoon DS
In preparation
- **Serial Monitoring of PTH Levels During Surgery for Hyperparathyroidism Further Optimizes Prediction of Treatment Outcome**
de Maat MF, van de Velde CJ, Fröhlich M, Kievit J
In preparation

Color Figures



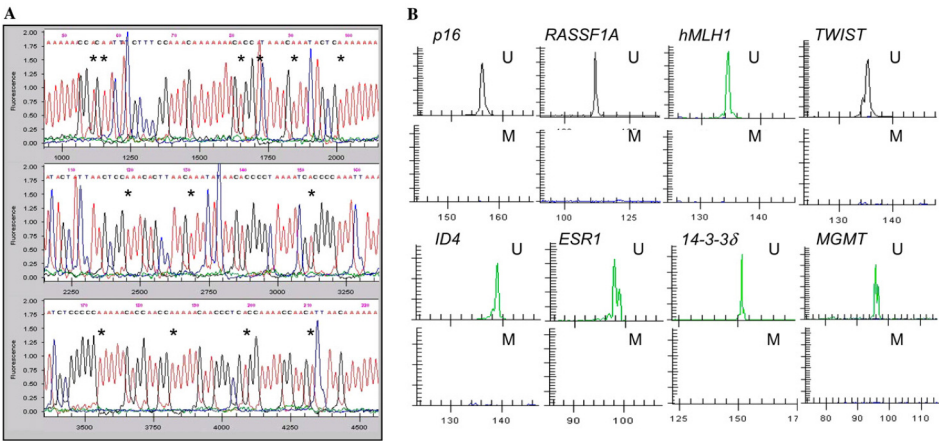


Figure 3. (A) SBM direct sequencing result at p16 promoter region on UUC in reverse direction. It is shown that all the cytosines of CpG dinucleotides in UUC were converted to uracils (shown as A in reverse sequence with *), representing that there was no methylation on CpG dinucleotides. (B) Methylated- and unmethylated-specific MSP for p16, RASSF1A, hMLH1, TWIST, ID4, ESR1, 14-3-3d, and MGMT on SBM UUC. Unmethylatedspecific peaks were observed in unmethylated-specific MSP in upper figures (U), but no peaks were observed in methylated-specific MSP in lower figures (M). The vertical axis represents the fluorescent intensity indicating the amount of PCR amplicon and the horizontal axis represents the PCR amplicon size.

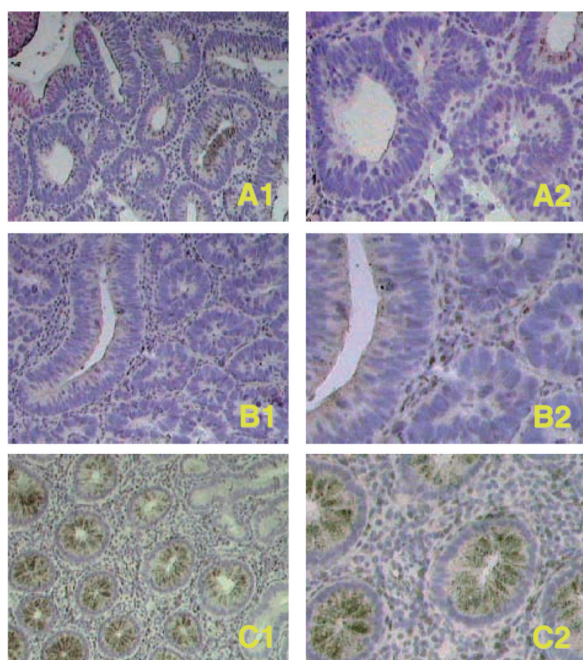


Figure 1 Microscopic photographs of three tissue sections after on-slide SBM treatment and hematoxylin staining. Magnification, 100 (A1, B1, and C1) and 200 (A2, B2, and C2).

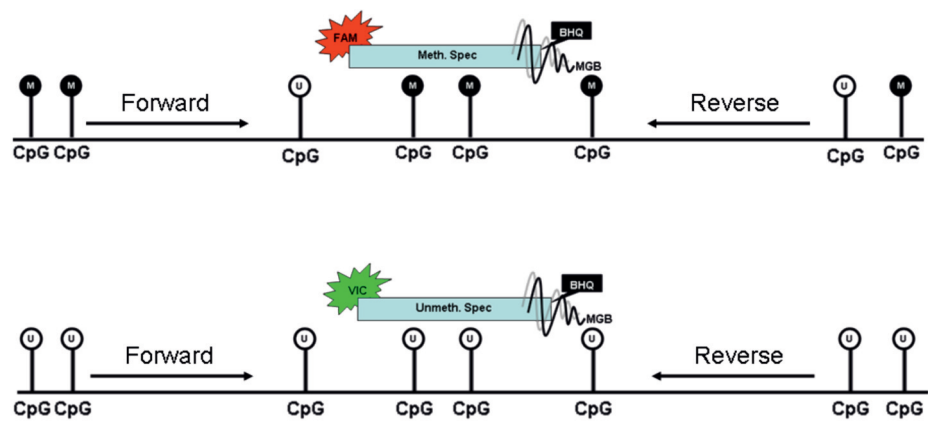


Figure 1. Schematic representation of the AQAMA assay. A universal primer set amplifies a target sequence. **A.** A methylation-specific probe with FAM-labeled reporter, BHQ, and MGB molecule recognizes sample DNA showing hypermethylation. **B.** An unmethylated-specific probe with VIC-labeled reporter, BHQ, and MGB molecule recognizes unmethylated sample DNA.

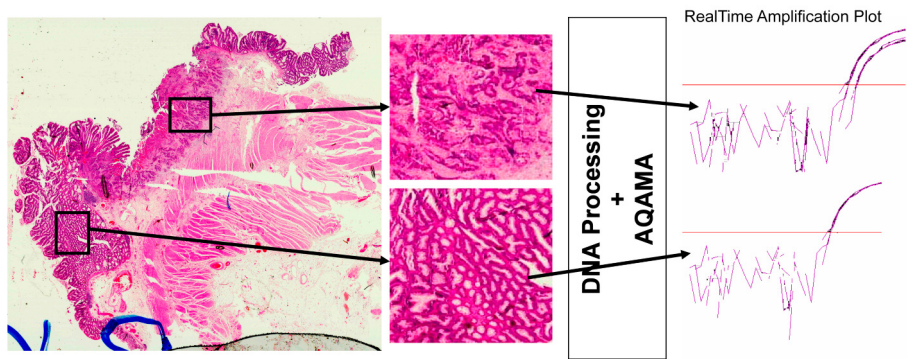


Figure 4 Schematic representation of histology-oriented tissue isolation followed by AQAMA. Left, AQAMA PCR plot. The adenomatous tissue component (bottom marked area) shows only unmethylated fluorescent signal (triplicate results), whereas the cancerous component (top marked area) shows both unmethylated and methylated fluorescent signal. Both signals are visualized here; however, in the raw data analysis, the CT is analyzed separately.

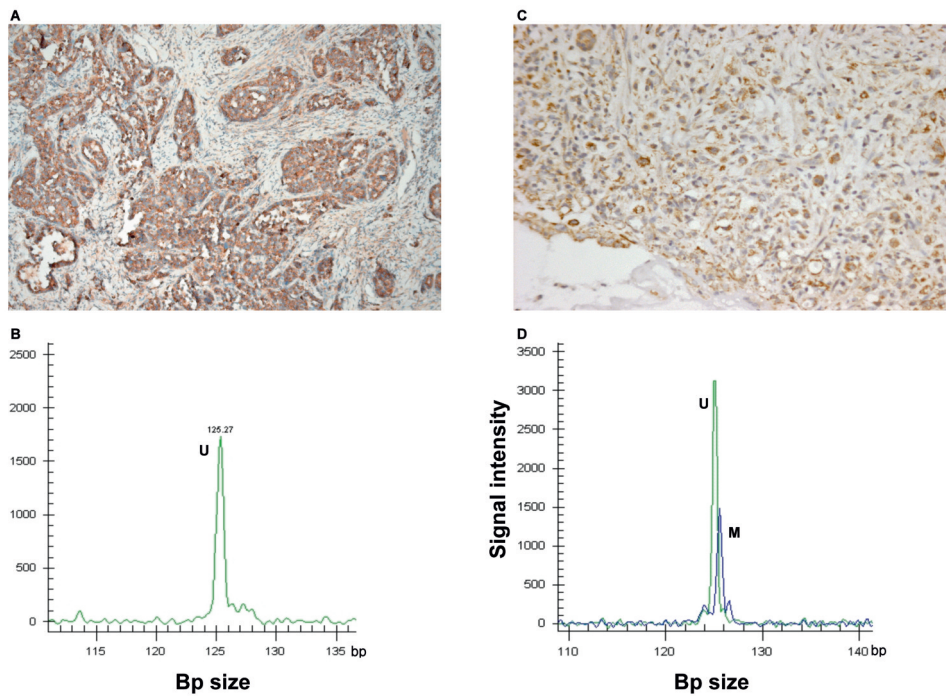


Figure 2. Representative cyclooxygenase-2 (COX-2) immunohistochemistry (IHC) results of primary gastric tumors with respective capillary array electrophoresis methylation-specific polymerase chain reaction (CAE-MSP) outcomes. *x*-axis of the CAE-MSP represents the fluorescent intensity (*M*, methylated product; *U*, nonmethylated product) indicating polymerase chain reaction amplicon. *y*-axis represents the product base pairs. (A, C) Nonmethylated primary gastric tumor. Strong cytoplasmic COX-2 protein staining in tumor cells. (B, D) Methylated primary gastric tumors show weak diffuse IHC staining.

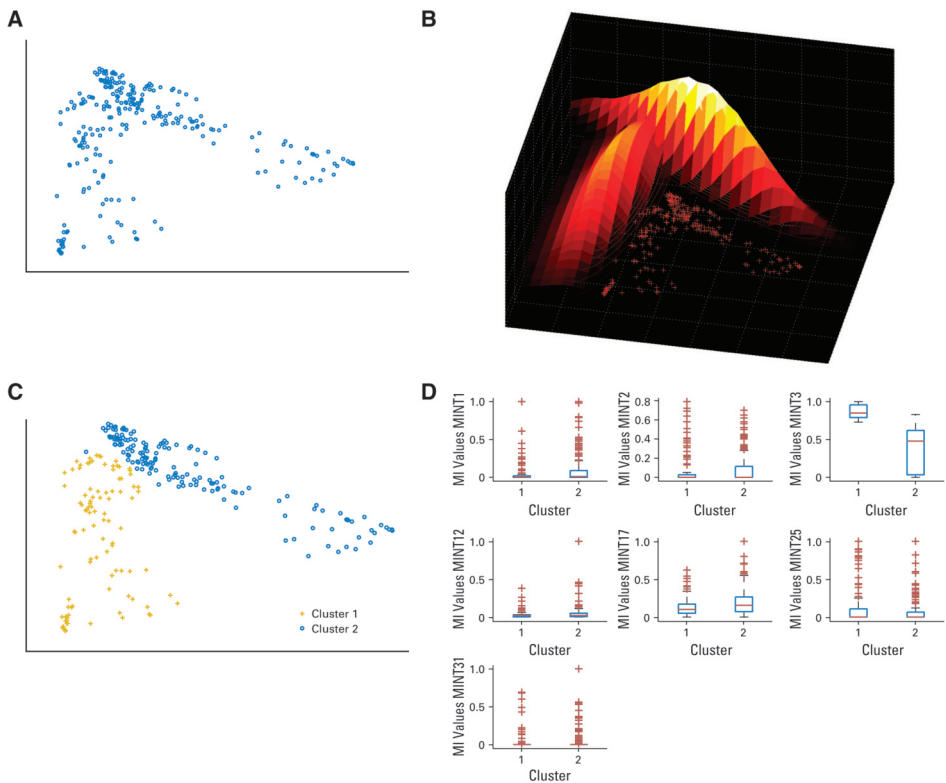


Figure 2. Methylated in tumor (MINT) locus methylation subclass identification. (A) multidimensional scaling [MDS] plot displaying the level of dissimilarity between all patients (MDS plot axes represent arbitrary units, and are therefore dimensionless). (B) Three-dimensional plot representing expectation maximization algorithm with a mixture of Gaussians analysis of the MDS plot coordinates showing Gaussian distribution (bell-shaped) of the two identified clusters. (C) MDS plot showing final cluster allocations for the patient population. (D) Box plots comparing the differences in methylation levels (MI) between cluster 1 and 2 for all MINT loci.

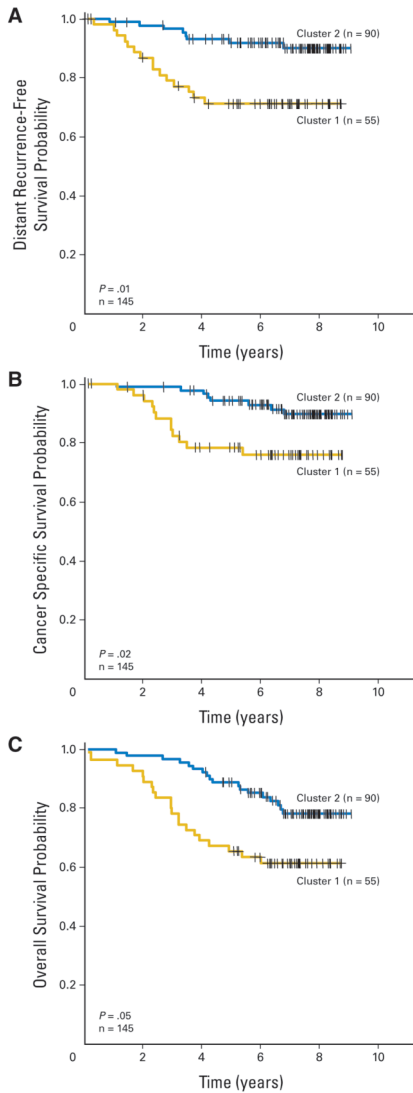


Figure 3. (A) Kaplan-Meier plots grouping analyzed node-negative total mesorectal excision trial patients into clusters 1 and 2 and comparing postoperative distant recurrence free survival probability. (B) Cancer-specific and (C) overall survival are plotted.

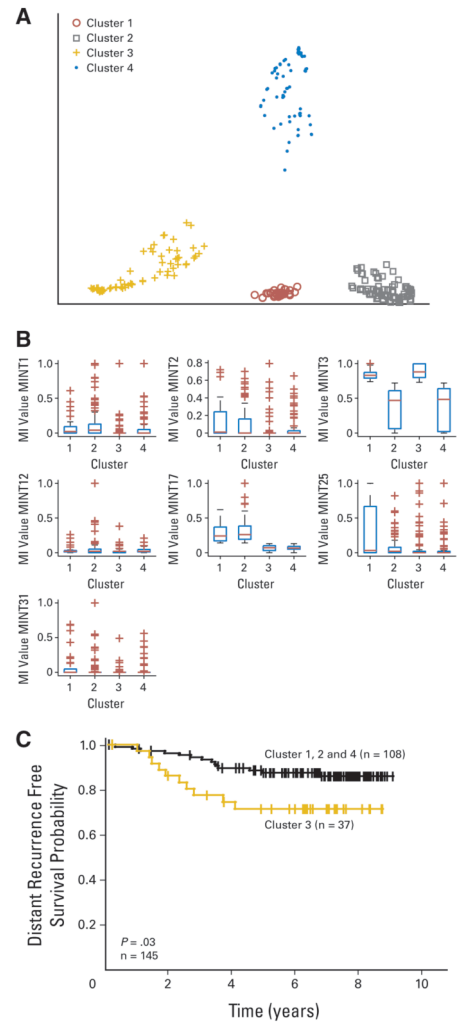


Figure 4. Random forest analyses using only MINT3 and 17 quantitative methylation data as input. (A) Multidimensional scaling plot showing the four clusters. (B) Box plots comparing the differences in methylation levels (MI) between the four clusters. (C) Kaplan-Meier plot illustrates the distant recurrence probability between high-risk cluster 3 and the combined clusters 1, 2, and 4.

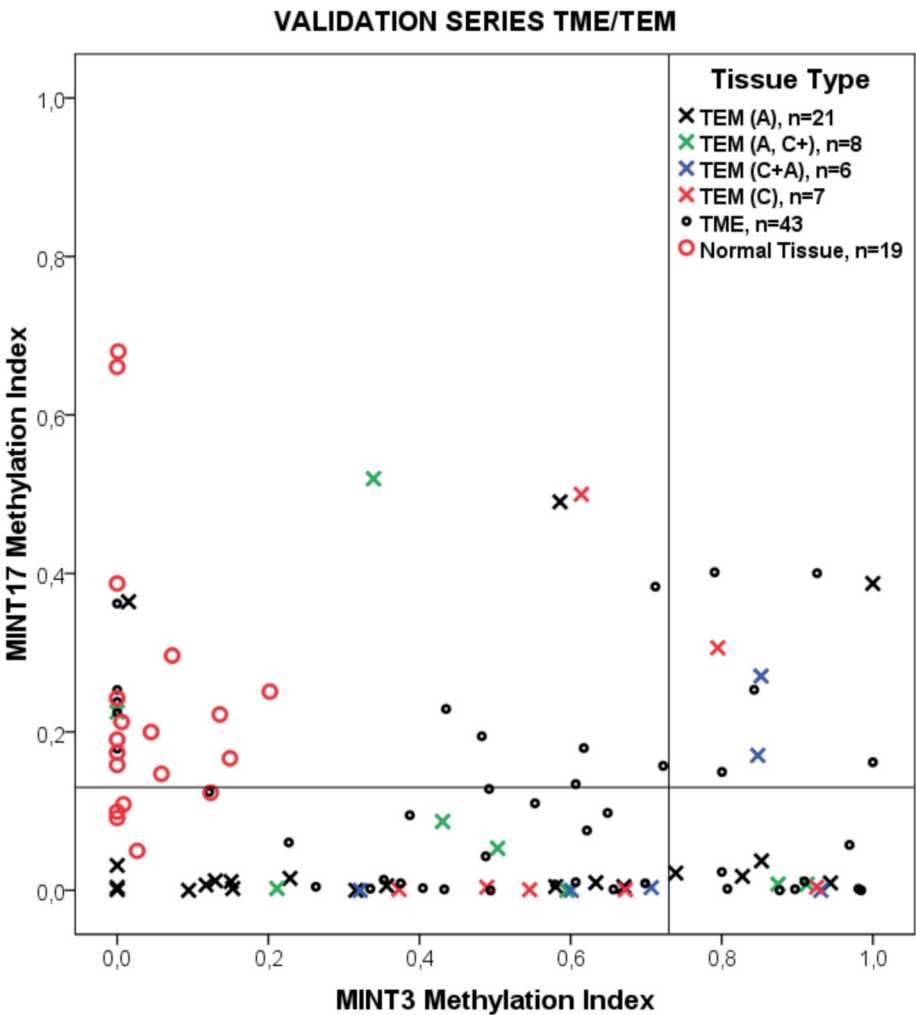


Figure 4. Two dimensional cut-off validation experiment results. Representation of MINT3 (X-axis) and MINT17 (Y-axis) methylation indices for patients of the TME trial, TEM study and 19 normal rectal mucosa samples. The horizontal and vertical reference lines represent the previously suggested cut-offs to separate the groups. For the TEM series, the following specimens were assessed: (A) adenoma with no carcinoma cells; (A, C+) adenoma with carcinoma cells; (C+A) carcinoma mixed with adenoma cells; and (C) carcinoma.

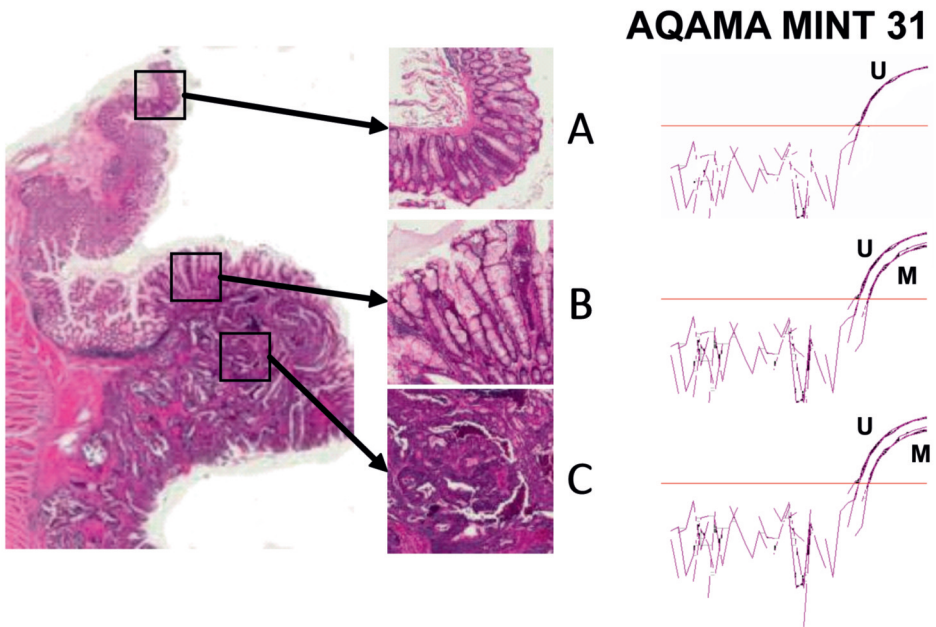


Figure 1: Representative examples of molecular analyses in normal (A), adenoma (B) and cancer (C) tissue. AQAMA (MINT31) real-time PCR results showing results in triplicate of exponential amplification of methylated (M) and unmethylated (U) dye probe signal. Y-axis represents signal intensity, X-axis represents PCR cycle number.

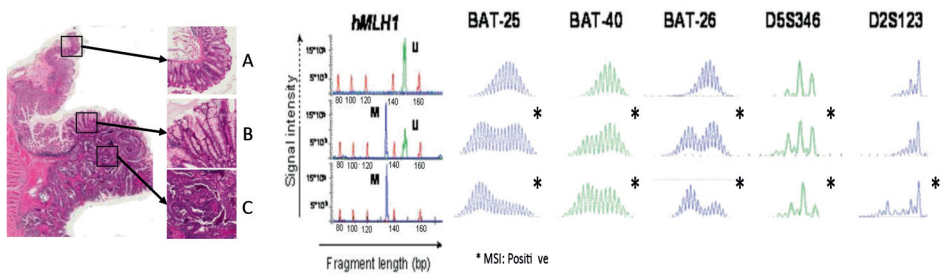


Figure 2: Representative examples of molecular analyses in normal (A), adenoma (B) and cancer (C) tissue. CAE results of amplified dye-labeled PCR products of 5 MSI biomarkers and *hMLH1* methylation-specific (M, in blue) and unmethylated-specific sequence (U, in green) primer sets. Red peaks represent the DNA ladder signal. The horizontal axis represents size of the PCR product in base-pairs (bp). The vertical axis represents arbitrary units of signal intensity of detected amplicons.

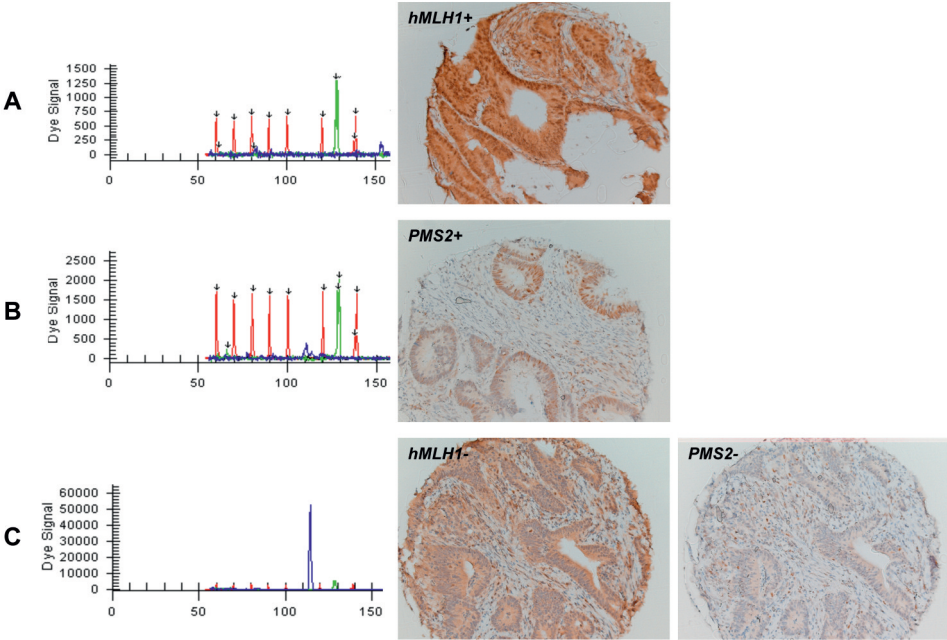


Figure 5: Representative IHC staining of hMLH1 and PMS2 proteins. In **A** and **B** examples of hMLH1 and PMS2, respectively, expressing CRC with their corresponding CAE-MSP results for hMLH1 showing unmethylated signal only. In **C**, an example of a CRC showing methylation of the hMLH1 promoter region with corresponding absence of nuclear staining of hMLH1 which is confirmed by underexpression of PMS2.

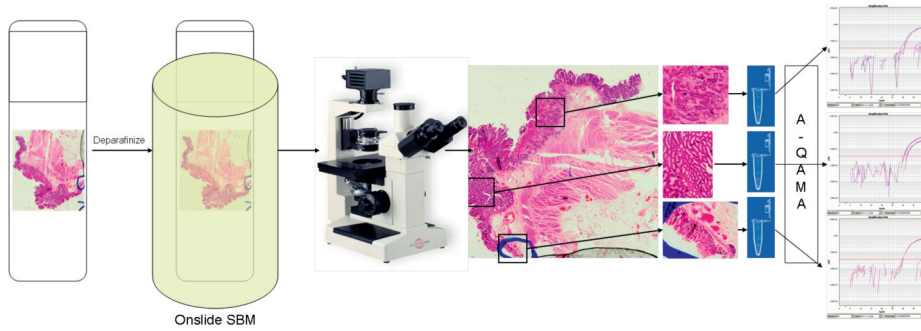


Figure 1: Schematic representation of the on-slide SBM procedure