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 gen 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 oncolo-logs started a randomized trial on surgery for gastric cancer comparing two levels of lymph node dissection. The successful surgical enterprise lead 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 developed 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.