Chapter 1

General introduction and thesis outline

1

Introduction

Colorectal cancer: treatment and prognosis

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

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

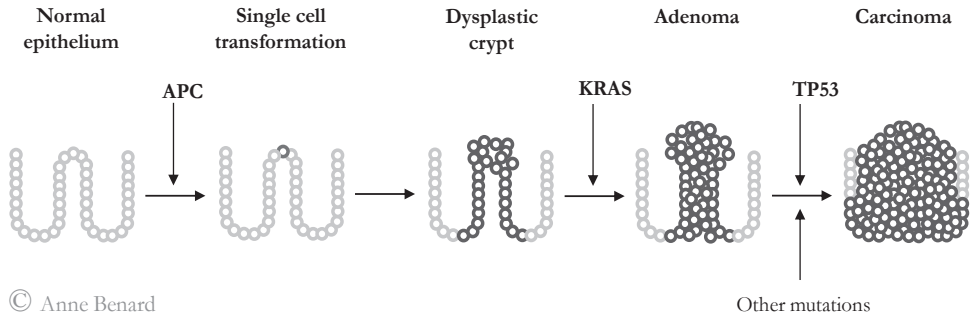
Biomarkers are biological markers that can be measured in for example blood or tumor tissue and can be used as indicators of pathological processes and hence provide information on the likely clinical outcome (prognostic biomarkers) or to measure the response to therapeutic interventions (predictive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

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

Genetic changes in colorectal cancer – history and current knowledge

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

Figure 1



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

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

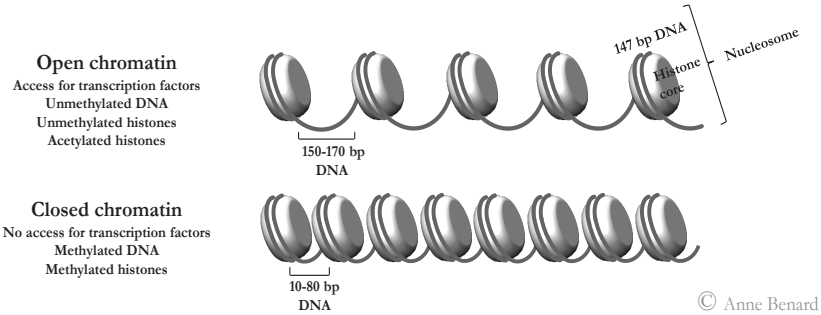
Epigenetic regulation of gene expression

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

DNA methylation

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

Figure 2



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

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

Histone modifications

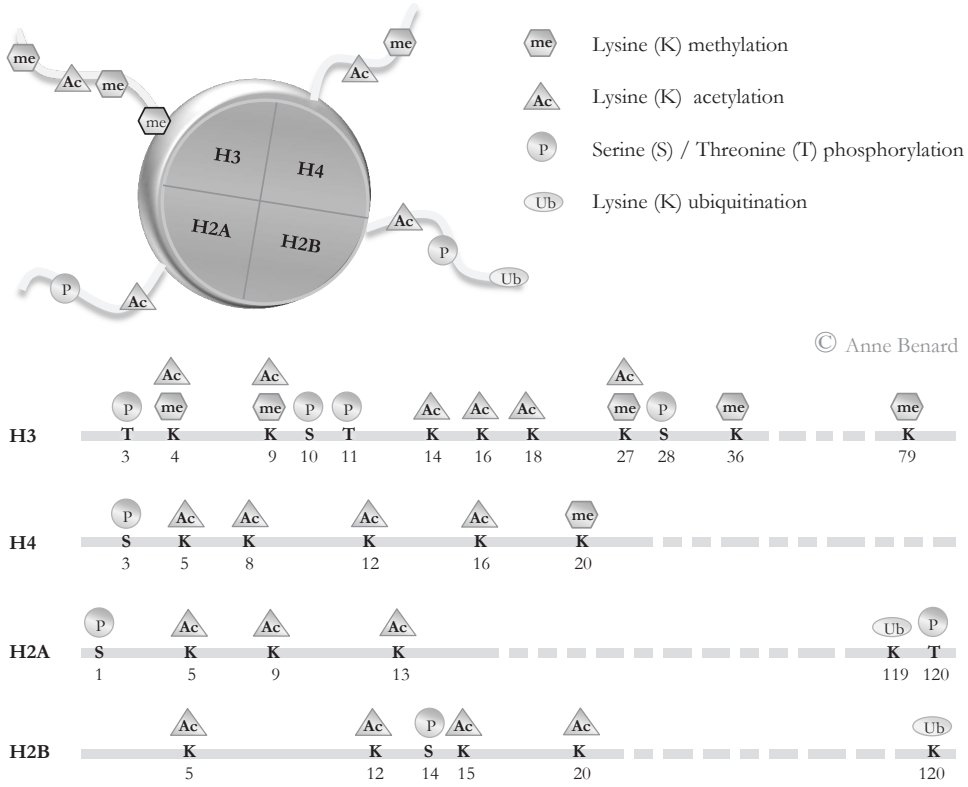
Together with DNA methylation, histone modifications are major factors in determining chromatin structure. Eight histone proteins, in four pairs of histones H2A, H2B, H3 and H4, constitute the core of the nucleosome (Figure 3), around which around 147 basepairs of the DNA are wrapped (47). Protruding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

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

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

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

Figure 3



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

Thesis aim and outline

Thesis aim

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

Thesis outline

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

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

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