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Title: Prognostic and predictive biomarkers in colorectal cancer. Towards precision medicine
Issue Date: 2015-01-08

CHAPTER 1

General introduction

INCIDENCE AND AETIOLOGY

Colorectal cancer (CRC) is the third most common cancer and is one of the major contributors to cancer-related deaths worldwide [1;2]. Approximately 20-25% of patients with CRC already have metastatic disease at the time of diagnosis and 20-25% of patients will develop metastases during disease progression as well, resulting in a 40-45% high mortality rate [3;4]. CRC can be divided in colon cancer, where the development of tumors ranges from the caecum to the sigmoid, and rectal cancer, that ranges from the rectosigmoid to the anus. Approximately one third of all colorectal cancers constitutes of rectal cancer.

CRC originates most often sporadically, is inherited in only 5% of the cases and evolves from benign pre-neoplastic lesions, such as adenomatous polyps or adenomas. The adenoma-carcinoma sequence, a series of well-defined histological stages, is responsible for progression of these benign lesions to malignant carcinomas [5]. Hanahan and Weinberg established six biological capabilities which tumors must acquire during the multistep development of human cancers, also called the hallmarks of cancer [6]. These hallmarks are sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death. More recently, they added two emerging hallmarks; reprogramming of energy metabolism and evading immune recognition and recognized the importance of the tumor-microenvironment, a repertoire of recruited normal cells around the tumor that contributes to the acquisition of these hallmarks as well [7].

Underlying these hallmarks are genome instability, which generates the genetic diversity responsible for the acquisition of these hallmarks, and inflammation [7]. In CRC, three major mechanisms of genetic instability responsible for tumor development and progression have been identified (Figure 1). The first mechanism is through mutations in DNA mismatch repair (MMR) genes, which results in a failure to repair errors that occur during DNA replication, followed by alteration of the length of short, repetitive DNA sequences, called microsatellites, that occur in the human genome. This failure leads to the microsatellite instability (MSI) phenotype and is the hallmark of the hereditary Lynch Syndrome [8]. In addition, in 12-15% of sporadic CRCs MSI has been found as well, but here hypermethylation of the *hMLH1* promotor has been associated with this MSI phenotype [8]. MSI tumors are more frequently right-sided, poorly differentiated, display more often the mucinous cell-type, show more peritumoral lymphocytic infiltration and are associated with an improved survival [9]. Second, most CRCs arise through the chromosomal instability (CIN) pathway, which is also involved in CRC pathogenesis. CIN is observed in 65-70% of sporadic CRC and is characterized by allelic losses (loss of heterozygosity), chromosomal amplifications, and translocations in CRC cells [9;10]. More recently, the existence of a new pathway for CRC development has gained attention.

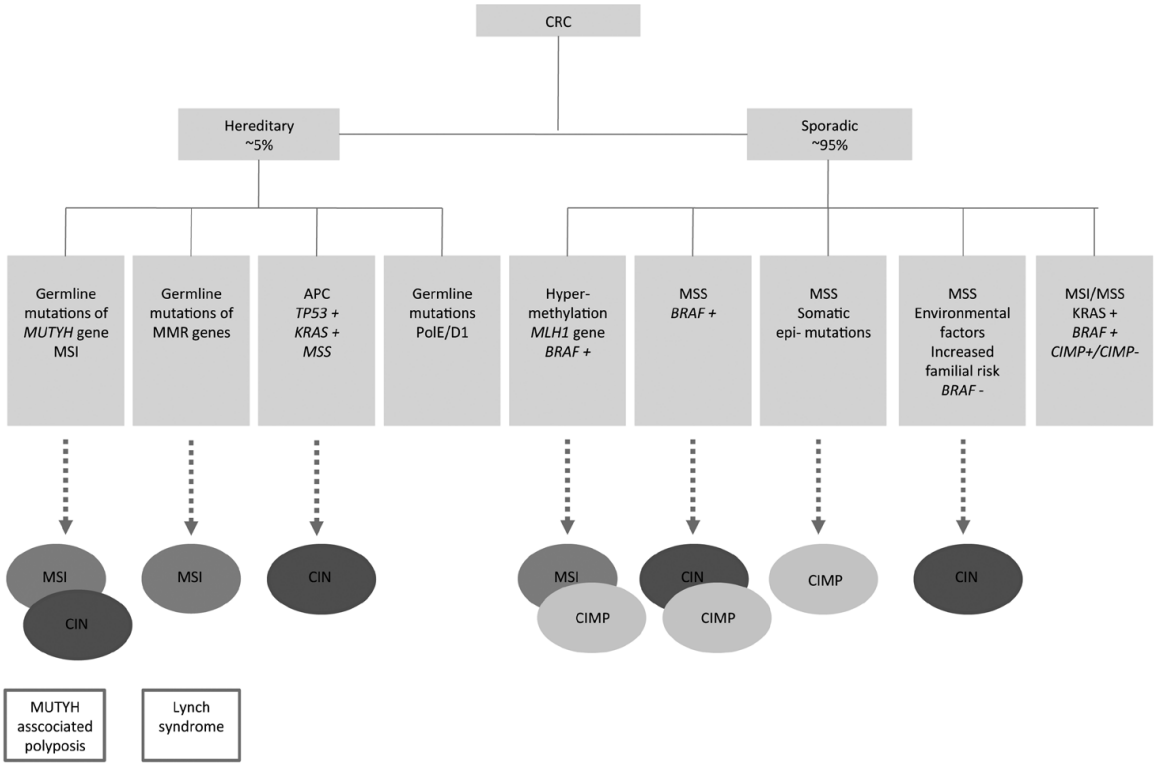

Figure 1: Global overview of major mechanisms of genetic instability responsible for tumor development and progression in CRC.
Abbreviations; CRC colorectal cancer, MSI microsatellite instability, MSS microsatellite stability, CIN chromosomal instability, CIMP CpG island methylator phenotype, BER base excision repair machinery, MMR mismatch repair, MLH1 MutL homolog 1, APC adenomatous polyposis coli, TP53 tumor protein 53, KRAS V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog, MUTYH mutY Homolog, PolE DNA polymerase ε, PolD1 DNA polymerase δ. + indicates mutation, - indicates wildtype.

These tumors are classified as having the CpG island methylator phenotype (CIMP), which involves the transcriptional silencing of tumor suppressor genes by hypermethylation of CpG islands of the promoter region of various genes [11]. Approximately one-third to one-half of all CRCs may evolve through this pathway [12].

Recently, three main molecularly distinct subtypes of colon cancer, each associated with unique clinical and molecular features, were demonstrated [13]. The first subtype demarcates the well characterized MSI/CIMP+ subset of colon cancers, which is mainly located on the right side of the colon. The second subtype, mostly left-sided, is largely devoid of MSI/CIMP+, but is typically associated with *KRAS* and/or *TP53* mutations, suggesting to represent the well-described CIN tumors. The third subtype is evenly distributed throughout the colon, enriched with histologically poorly differentiated tumors, heterogeneous with respect to MSI or MSS and CIMP status, and contains a large proportion of *KRAS* and *BRAF* mutations as well. This subtype is associated with a poor prognosis and poor response to anti-epidermal growth factor receptor (EGFR) therapy. The enhanced expression of epithelial mesenchymal transition (EMT) and matrix remod-

eling in these tumors provides a possible explanation for their poor prognosis and why these tumors metastasize more frequently as compared to subtype 2. Furthermore, evidence showed that this subtype is highly related to serrated adenomas as serrated precursor lesions are thought to progress in this subtype of colon cancer [13].

TREATMENT

Treatment choices are nowadays influenced by the tumor, node and metastasis (TNM) classification, which aims to provide an exact prediction system for prognosis, to guide therapy choices and to create uniformity in cancer language [14;15]. The survival of CRC patients largely depends on disease stage at diagnosis and varies widely between stages. In stage I, a five-year survival rate of 93.6% is seen, which drastically drops to 8.1% in stage IV patients [16].

The treatment of colon cancer comprises surgical resection of the primary tumor and regional lymph nodes. The last two decades, the role of adjuvant chemotherapy has gained importance and resulted in the introduction of a chemotherapy regimen in the Netherlands, consisting of oxaliplatin, fluorouracil and leucovorin, in stage III (lymph positive) and high-risk stage II colon cancer patients [17].

Nowadays, patients with rectal cancer are treated with pre-operative (chemo) radiation (5x5 Gy) followed by surgical resection using the total mesorectal excision (TME) technique. Before the introduction of the TME technique the 5-year local recurrence rate of rectal cancer with conventional surgery was over 20% [18]. The last decades these local recurrence rates have decreased drastically, mainly influenced by the introduction of the TME technique and the introduction of pre-operative radiotherapy since the Dutch TME trial, which investigated the effect of short-term preoperative radiotherapy in combination with TME surgery compared to TME surgery alone between 1996 and 2000 [19;20]. The role of adjuvant chemotherapy in rectal cancer is still debatable. The use of adjuvant chemotherapy in patients not treated with pre-operative radiotherapy or chemotherapy seems beneficial [21], however, in patients treated pre-operatively no survival benefit has been reported [22-24].

ASPIRIN TREATMENT

The last decade aspirin is gaining ground in the treatment of CRC patients. There is a significant amount of evidence demonstrating that aspirin has anti-cancer effects [25-32]. The first evidence comes from large cardiovascular prevention trials assessing the cardiovascular benefits of aspirin [29-32]. In a pooled analysis of five large trials aspirin

taken for several years at doses of at least 75 mg daily has shown to reduce long-term incidence and mortality due to CRC [29]. Furthermore, aspirin showed to significantly reduce adenoma formation in patients with a history of CRC [33]. More recently, aspirin has shown to be beneficial as adjuvant treatment as well. Aspirin taken after diagnosis significantly improved overall survival and colorectal cancer-specific mortality in patients with CRC [26;28;34]. At the moment, three recently started trials, ASCOLT in Asian CRC patients, the Big A trial in lung cancer patients and the Add Aspirin trial in colorectal-, breast-, upper gastrointestinal- and prostate – cancer patients, investigate the role of aspirin as adjuvant treatment (http://clinicaltrials.gov).

The exact mechanism by which aspirin exerts its activity is not completely understood. Direct inhibition of the cyclooxygenase (COX) family of enzymes involved in prostaglandin synthesis has been attributed to the protective activity of aspirin. The COX-2 enzyme is strongly and rapidly induced in response to mediators of inflammation, growth factors, cytokines, and endotoxins; and its expression correlates with increased cell proliferation and tumor promotion [35]. Aspirin can decrease the production of potentially neoplastic prostaglandins arising from COX-2 mediated catalysis of arachidonic acid [36].

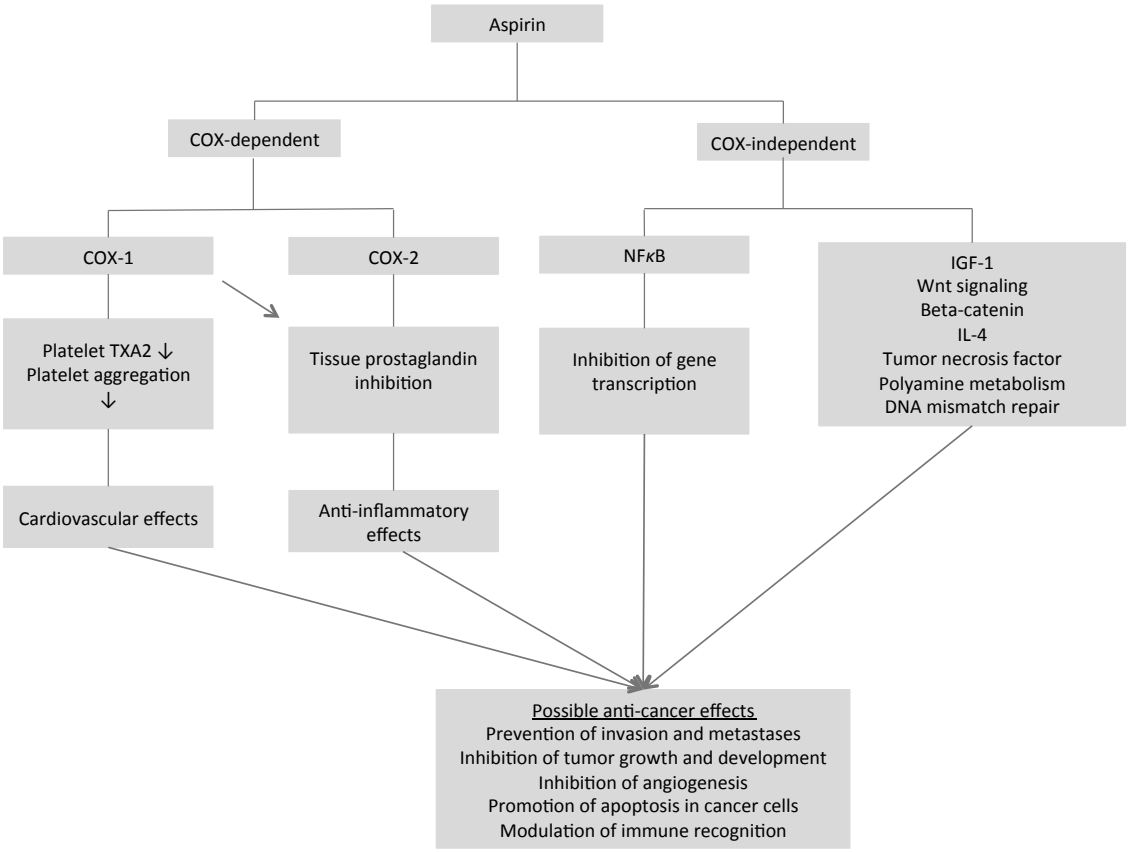

Figure 2: Global overview of possible pathways responsible for the anti-cancer effects of aspirin.
Abbreviations; COX: cyclooxygenase,TXA2 thromboxane A2, NFκB nuclear factor-κB, IGF-1 insulin growth factor 1. Partly based on Langley *et al*, BJC 2011; 105,1107-1113.

However, research has shown that aspirin has a much broader range of downstream effectors as well, such as NF-κB, insulin-like growth factor I (IGF-1), and the inhibition of Wnt-signaling and stem cell growth possibly as the result of enhanced beta-catenin phosphorylation [27;37-39] (Figure 2).

Studies trying to unravel the anti-cancer effects of aspirin thus far have been inconsistent. Possibly, more than one mechanism is responsible for the anti-cancer effects of aspirin. It is also plausible that different molecular mechanisms are responsible for the beneficial effects of aspirin on CRC incidence (prevention) than on already established CRC (therapy).

In the preventive setting of CRC COX-2 might play an influential role since regular aspirin use has shown to be associated with a lower risk of CRCs that overexpress COX-2, but not CRCs without COX-2 overexpression [26]. Also, inhibition of WNT/cadherin-associated protein β1 signaling (CTNNB1 or β-catenin), one of the most essential oncogenic pathways in CRC, has been described to reduce the risk for CRC. Aspirin inhibits this CTNNB1 signaling pathway COX-dependently but also through COX-independent pathways by directly inducing phosphorylation and subsequent degradation of CTNNB1 [40]. More recently, a study showed that aspirin use stabilizes DNA methylation at promotors of genes controlling critical cancer pathways. Age dependent methylation was suppressed in aspirin users and long-term aspirin use was associated with a more than 50% suppressed rate of methylation when compared with nonuse. Aberrant DNA methylation in gene promotors has been associated with aging and cancer [41].

In the first study investigating the molecular mechanisms responsible for the therapeutic effect of aspirin after a CRC diagnosis, COX-2 expression was mentioned to play a major role [26]. The survival benefit with aspirin use after diagnosis in CRC was associated with COX-2 expression of the tumor. A much lower risk of CRC-specific and overall mortality with tumors that overexpress COX-2 was found.

Research from the same group has also shown that aspirin may suppress cancer-cell growth and induce apoptosis by blocking the phosphatidylinositol 3-kinase (PI3K) pathway upstream of COX-2 [42]. This pathway plays an important role in carcinogenesis [43]. Mutations in *PIK3CA* (gene encoding for phosphatidylinositol-4,5-biphosphonate 3-kinase, catalytic subunit alpha polypeptide) are found in approximately 15 to 20% of colorectal cancers [44].

Furthermore, COX-1 might also be responsible for the improved survival of aspirin users in CRC, since aspirin influences platelet aggregation through COX-1 inhibition [45]. Recent evidence suggest that platelets may play an important active role in promoting metastasis by active signalling to tumor cells through the TGF-β and NF-κB pathways resulting in a prometastatic phenotype that facilitates tumor cell extravasation and metastasis [46]. Aspirin has shown to inhibit the activation of NF-κB [47].

Finally, it has been shown that IL-4 expression is essential for the resistance to DNA damage-induced apoptosis of colon cancer stem cells (CSCs) [48]. CSCs are also resistant to the cytotoxic effect of chemotherapy. It has been shown that IL-4 confers colon CSCs with resistance to apoptosis [48]. Consistently, treatment with IL-4Ra antagonist or anti-IL-4 neutralizing antibody strongly enhances the antitumor efficacy of standard chemotherapeutic drugs through selective CSCs sensitization. Notably, aspirin inhibits IL-4 gene expression [49]. Based on the above observations, it is plausible that aspirin may both act as a preventive agent in CRC onset by modulating the Wnt pathway in CSCs, but also as adjuvant treatment by increasing CSCs' sensitivity to conventional chemotherapy regimens.

PROGNOSTIC AND PREDICTIVE BIOMARKERS IN CRC

To date, tumor location and tumor stage have majorly influenced treatment decisions. However, new insights and advances in the molecular biology of CRC have started to influence prognostication and treatment decisions. Molecular mechanisms responsible for tumorigenesis are likely to influence clinical outcome [6]. Also, research has shown that approximately 20-25% of patients with lymph-node negative stage II colon cancer, which were not recommended adjuvant treatment based on TNM stage, suffer from recurrent disease within 5-years of follow-up [50]. The TNM stage is therefore not an optimal tool for prognostication and treatment allocation, especially in high-risk stage II patients, and needs to be supplemented with additional biomarkers that can improve the current staging and treatment allocation criteria substantially.

By investigating biomarkers that reflect tumor growth and metastatic potential, a more accurate prediction on prognosis and treatment benefit based on underlying biology can be made. Predicting the clinical behavior of a tumor through a combination of clinical, pathological and biological characteristics may lead to a well-targeted treatment in the individual patient. Evading immune recognition, sustaining proliferative signaling and resisting cell death are important mechanisms that cancer cells acquire during further tumor development [7] and are therefore studied in the research described in this thesis.

OUTLINE OF THIS THESIS

The aim of this thesis was to define prognostic and predictive biomarkers in colorectal cancer for improved risk stratification and treatment benefit in the individual patient, with the introduction of precision medicine in the near future as the ultimate goal. By

definition, precision medicine is a multi-faceted approach to medicine that integrates molecular and clinical research with patient data and clinical outcome, and places the patient at the center of all elements. This thesis is divided in three parts. In ***Part one*** prognostic biomarkers in CRC are investigated, in ***Part two*** aspirin treatment and related predictive biomarkers for aspirin treatment benefit in colon cancer are investigated and finally, in ***Part three***, the use of predictive and prognostic biomarkers in clinical practice, its utility and the road to precision medicine are discussed.

The last two decades, research has shown that the immune system has a substantial effect on tumor growth and metastasis [51]. Tumors are thought to be 'edited' through a Darwinian selection process in poorly immunogenic tumor cell variants able to evade immune recognition and consequently growth progression [52-55]. Several mechanisms in the tumor contribute to this process. First, downregulation of human leukocyte antigen (HLA) class I expression, which minimizes the level of tumor-associated antigen (TAA) expression by tumor cells, followed by less immune recognition and subsequently less destruction by cytotoxic T-cells (CTL) [56]. Second, expression of non-classical HLA class I molecules (HLA-E and HLA-G) on the tumor cell surface. HLA-E is regularly expressed in various healthy tissues and correlates with HLA class I expression [57]. In contrast, HLA-G is rarely expressed in healthy tissues but has been frequently observed in tumors [58]. Both have been associated with inhibition of natural killer (NK) cell recognition resulting in further escape from immune recognition [58;59]. Third, attraction of immunosuppressive regulatory T cells (Tregs) into the tumor micro-environment, which suppress the activity of CTL [60;61]. Conflicting results have been described for the association between expression of these markers and prognosis in CRC patients, possibly due to the use of different patient cohorts and the investigation of solely one marker. Research has shown a complex relationship between different immune markers, highlighting the need for combined marker analysis [62-64]. Therefore, in **Chapter 2** we evaluated the association of these immune markers, separately and combined, with prognosis in colon cancer patients. We performed the same analysis in rectal cancer patients to investigate differences in immune escape mechanisms between colon- and rectal cancer in **Chapter 3.**

Deregulation of the proliferative signaling pathway and deregulation of the apoptotic pathway are also two important hallmarks of tumor development, which disturb tissue homeostasis and balance [6]. Previous studies have shown contradicting results with respect to the relation of apoptotic - or proliferation levels in tumor specimens and patient outcome in CRC [65-68]. In **Chapter 4** we therefore investigated if the combined analysis of these two processes would better reflect tumor aggressiveness.

Over the last decades the public health sector witnessed a vast and rapid development of genomic profiling techniques, with the promise of precision medicine as a strong driving force. Prediction of pathway deregulation coupled to molecular target identification using genome wide approaches may provide an opportunity to guide

treatment [69]. Since various molecular pathways are involved in carcinogenesis, multigene assays might give a more reliable insight in tumour biology and risk of recurrence than single-gene analysis. One of those multi-gene assays is the Onco*type* DX Colon Cancer Recurrence Score (RS) (Genomic Health, Redwood City, CA, USA), which measures the expression of 12 genes and was validated as a predictor of recurrence risk in stage II colon cancer patients [70;71]. Validation of this multi-gene assay in rectal cancer has been performed and described in **Chapter 5.**

In ***Part two*** of this thesis, the benefit from aspirin treatment in colon cancer is described. In **Chapter 6**, this benefit was investigated in older colon cancer patients. Recent studies have shown that regular use of aspirin after diagnosis was associated with longer survival among patients with mutated- *PIK3CA* CRC, but not among wild-type *PIK3CA* tumors [44], and among patients who express high tumor levels of COX-2 [26]. In **Chapter 7** we showed that colon cancer patients only benefit from aspirin treatment when these patients expressed HLA class I on their tumor cell surface. The aspirin benefit on survival was not associated with *PIK3CA* or COX-2 expression in our cohort.

In ***Part three*** of this thesis the use and introduction of biomarkers in clinical practice influencing precision therapy (**Chapter 8**) and the impact of genomic profiling on surgery (**Chapter 9**) are discussed. Finally, an overall summary and discussion of the data presented in this thesis are provided in **Chapter 10**.

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