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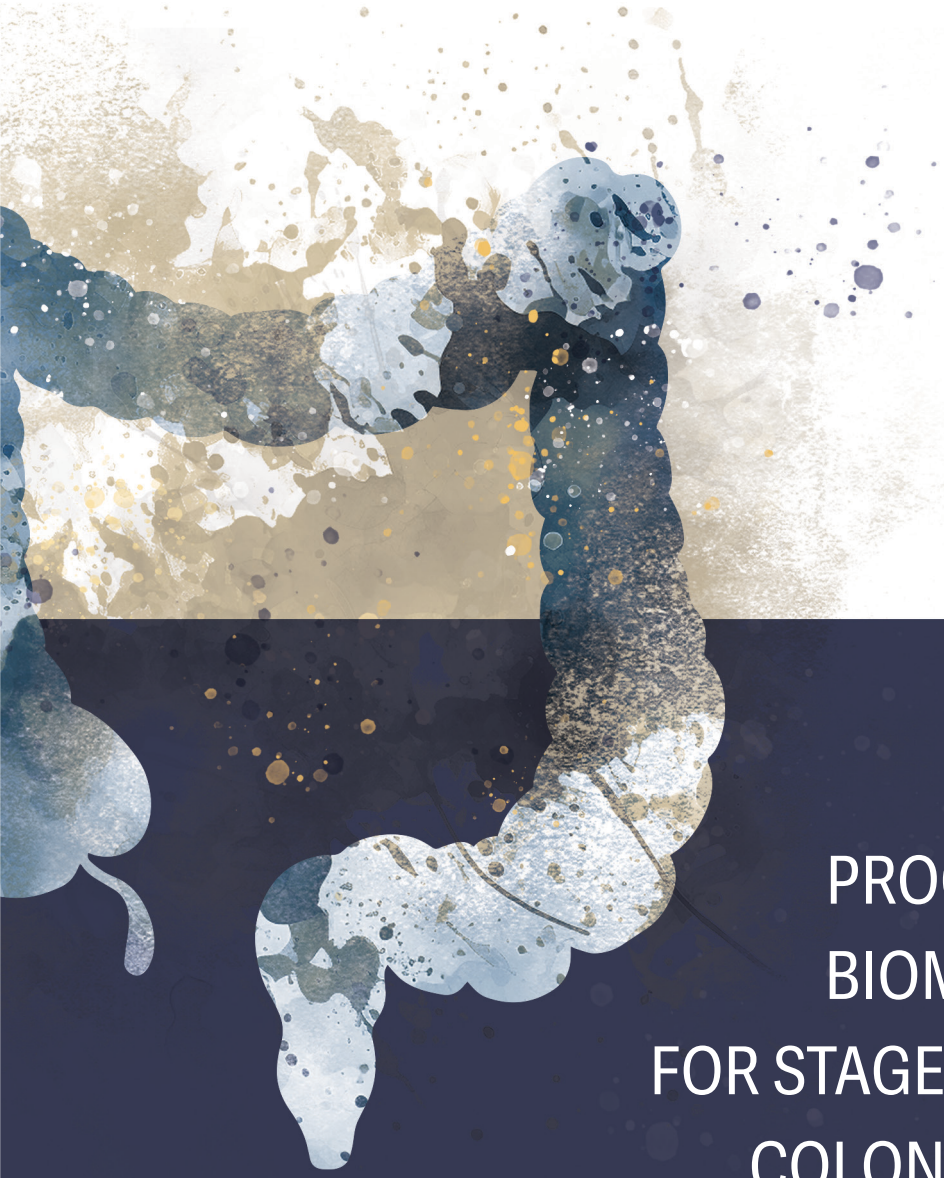

PROGNOSTIC BIOMARKERS FOR STAGE II AND III COLON CANCER

Sjoerd Hendrik den Uil

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CHAPTER 1

Introduction, aim and outline of the thesis

1. GENERAL INTRODUCTION TO CANCER

1.1 Etymology

Cancer is defined as a disease caused by an uncontrolled division of abnormal cells with the potential of invasion in surrounding-, or spreading to distant, tissues. It has been written about since as early as the ancient Egyptians. Hippocrates also referred to cancer as Karkinos, meaning crab in Greek. The hard shell and its feet reminded him of cross sections of solid tumours with sprouting blood vessels. The German word for cancer (Krebs) also refers to this metaphor, and a crab is still used as the symbol for cancer in the logo of a Dutch foundation that aims to fight cancer.

1.2 Development

Cancer is characterized by a disturbed balance of proliferation versus programmed cell death, leading to expansive cell division. Damage to the DNA is considered to be at the base of this disturbance, although DNA damage itself is a natural phenomenon that happens tens of thousand times a day for each cell [1]. However, when repair mechanisms are impaired, the amount of un-repaired DNA-damage increases, allowing faulty DNA to reproduce. However, only an accumulation of certain alterations lead to cancer development though, and usually several specific mutations in regulator-genes are required before a normal cell will actually transform into a cancer cell, the so-called multiple-hit hypothesis [2].

1.3 Genetics

Two general types of cancer-related genes are tumour suppressor genes (loss-of-function) and proto-oncogenes (gain-of-function). When mutations are present in a proto-oncogene, or if there are simply too many copies of this gene, the cell may show out-of-control growth. Suppressor genes contribute, in a physiological setting, to correction of DNA abnormalities, the slowing down of cell division and apoptosis. Mutation of these genes may lead to inactivation of these processes and disturbance of the balance of cell homeostasis, and subsequently contribute to uncontrolled cell division and eventually the formation of cancer.

DNA damage and finally cancer may be provoked by environmental or exogenous factors, of which lifestyle factors (i.e. alcohol and tobacco) and natural exposure to UV-light, radiation and certain viruses are well known. Furthermore, the influence of workplace- and home related exposures, pollution and of course medical treatments are described. Examples of cancers with well-known exogenous risk factors are localized in the oropharynx, liver, oesophagus, skin and lung [3]. These exogenous factors might contribute differently in different parts of the world, and therefore lead to a variability in cancer registries from over the world [3]. Endogenous agents also are capable to contribute to DNA damage. In inflamed intestinal epithelium for instance, reactive oxygen species are formed by macrophages and neutrophils [4], and in people with a diet high in fat, carcinogenic bile salts reach the colon [5]. Only for a few types of cancer it is possible to determine the specific initial cause. Usually more factors are involved, contributing in some way to its development.

Besides all these risk- and inducing factors, key point in the development of cancer is an insufficient repair mechanism for damaged DNA. Impaired repair mechanisms allow the accumulation and the survival of damaged DNA in a cell. First, damaged DNA might prevent the cell to transcribe the affected gene, inhibiting that function. Second, during replication, un-repaired damaged DNA may lead to incorrect base insertion in the complementary strand, thereby functionally influencing its daughter cells.

Eventually, unrepairable damage may lead to senescence or apoptosis of that cell. Alternatively, the cell can enter a third state, i.e. unregulated cell division, thereby increasing the risk of cancer formation. The actual risk for cancer development may vary between the different repair genes. Mutation of for instance p53, the guardian of the genome, results in a 100% lifetime risk of cancer. Examples of other inherited DNA repair gene mutations are BRCA1, BRCA2 and FANC.

1.4 Epigenetics

In addition to genetic abnormalities, epigenetic alterations may lead to reduced or absent expression of (repair) genes. Epigenetics was originally defined as 'the causal interactions between genes and their products, which bring the phenotype into being' [6]. It does not describe the effect of changes in the actual sequence of nucleotides, but in the way the genome translates to gene expres-

sion and/or phenotypes. This is an essential mechanism in development and maintenance of gene expression patterns, both physiological and in cancer, and disruption of these processes can result in altered function and malignant transformation [7]. Knowledge of these epigenetic mechanisms provided additional understanding to cancer, that was originally known to be of genetic origin [8]. The advantage of the increasing insight in this field, is that epigenetic changes can be reversible, and can return to their physiological function when treated duly [9, 10].

Mechanisms of actions include methylation, histone modification, nucleosome positioning and histone variants [11]. These mechanisms are indispensable for the development and regulation of gene expression, and the combination of epigenetic patterns determines cell fate and gene activity. Embryonic stem cells for instance, having the ability to differentiate depending on the localization in the body, maintain an epigenome mandatory for developmental processes. Already differentiated cells show an epigenome directed more towards maintenance [11-13].

DNA methylation, as the most extensively studied epigenetic mechanism, is a process where a methyl group (CH3) binds to cytosine (usually), forming methylcytosin, thereby changing the activity but not the sequence of that DNA segment [11]. The methyl group causes tighter coiling of DNA around the histone, preventing transcription. It provides a way of controlling or regulating transcription of the genome, hereby influencing cellular processes [7]. Methylation in humans is almost exclusively found in CpG dinucleotides, where usually cytosines of both strands are methylated. CG enriched areas in the DNA are called CpG islands, and around 70 % of the promoter regions contain such CpG islands [14].

A methylated status of such a promoter region, prevents transcription of that particular gene, and is therefore seen in non-coding DNA segments. It results in transcriptional silencing and contributes to chromosomal stability [15]. On the contrary, an unmethylated (or hypomethylated) status enables transcription [16], hereby potentially leading to chromosomal instability due to loss of imprinting and reactivation of transposable elements [17]. In cancer, both hyper- and hypomethylation occur, although usually in different DNA sequences

[18]. A combination of (hyper)methylation of tumour suppressor genes and hypomethylation of oncogenes may contribute to the development or progression of cancer [19].

1.5 Biological requirements

In order for a tumour to further develop and maintain once formed, it has to acquire additional biological functional features. These features include induction of angiogenesis, resistance to cell death, sustaining of proliferative signalling, evasion of growth suppressors, replicative immortality and the activation of invasion and metastasis, as summarized by Hanahan & Weinberg [20]. This paper also reviewed more recent hallmarks such as evading immune destruction and the deregulation of cellular energetics. These events can only occur when given the right support from surrounding tissues. This support is provided by tumour-associated stroma, that is formed by extra-cellular matrix, leucocytes, endothelial cells, tumour associated fibroblasts, etc. Instead of passively surrounding tumour cells, this stroma is actively stimulated by tumour cells, and on its turn stimulating and controlling the tumour micro environment, both locally and systemically, thereby also contributing to niche formation for metastasis [21, 22].

2. INTRODUCTION TO COLON CANCER

2.1 The numbers

Colorectal cancer (CRC) still ranks third in the number of new cases (10%) and second in the number of deaths (9.4%) among cancers worldwide [23]. In 2020, over 1.9 million new CRC patients were identified, with over 900.000 deaths. In the Netherlands CRC is ranked similarly. In 2021 there were over 9000 new colon cancer patients, approximately 7,5 % of all new cancer diagnoses, of whom the majority was older than 55 years. Males are slightly overrepresented in this nation-wide registry (4784 males with colon cancer in 2021, and 4450 females). The effect of the COVID-19 pandemic on these numbers is yet unknown. Over 3000 patients died of colon cancer in 2019 and 2020 in the Netherlands, and mortality increases with age, with similar rates for both genders [24].

2.2 Pathogenesis

In the first section of this introduction, development of cancer in general was discussed briefly. In this section, pathways involved in CRC are reviewed. CRC can be defined as a transformation of the normal colonic (or rectal) epithelium to a precancerous lesion and, eventually, to an invasive carcinoma (adenocarcinoma). Adenocarcinomas represent >90% of all CRC, however alternative histological subtypes are known [25, 26]. The adenoma-carcinoma sequence is mediated by a variety of genetic and molecular pathways, i.e. chromosome instability, microsatellite instability and/or epigenetic instability.

Chromosomal instability (CIN) is the mechanism leading to alterations in the number and structure of chromosomes and failure to maintain euploidy, leading to aneuploidy. A well-known example of aneuoploidy is Down syndrome (trisomy 21), however congenital aneuploidy is often lethal in utero. CIN is able to induce a range of genomic changes, from subtle to massive. For tumours, CIN is an efficient way to generate several karyotypes, thereby enhancing the chance of a viable karyotype. This 'natural selection' is vital in tumour development, as the tumour experiences all kinds of stress that needs addressing in order to survive. Mutations of proto-oncogenes and tumour suppressor genes (first APC, later KRAS, TP53, PIK3CA and TGF-β) may further fine-tune the development by activation of pathways crucial for CRC initiation and progression. Loss of APC leads to decreased binding to β-catenin and to subsequent decreased inhibition of Wnt-signalling, that regulates growth, apoptosis and differentiation [27]. APC mutations are found in approximately 60 % of colon cancers. Loss of SMAD (subtypes 2 and 4) is also frequently seen (60 %), and is related to the TGF-β signalling pathway, which is important in regulating growth as well as apoptosis. Activation of KRAS and PIK3CA results in induction of MAPK, thereby promoting cell proliferation [28]. Functional loss of TP53 is seen more frequently later in the adenoma to carcinoma transition, with up to 75 % in colorectal cancers compared to 4-26 % in adenomas [29]. TP53 is known to increase expression of cell-cycle genes and later, when there is too much genetic damage, it enhances pro-apoptotic genes [30]. CIN can be detected in up to 70-85 % of the sporadic colorectal cancers [27, 31-34].

Microsatellite instability (MSI) is less frequently observed, in approximately 15 % of the non-metastatic CRC's, and 3-5 % of the metastatic CRC's. Microsatellites

are nucleotide repeat sequences scattered throughout the genome (coding and non-coding), and instability is characterized by discrepancy in the amount of nucleotide repeats compared to the germline, through insertions and deletions. Due to failure of DNA mismatch repair (MMR), these insertions and deletions may accumulate and this leads to this form of genomic instability and additive mutations throughout the genome (a "hypermutator" phenotype).

Hereditary non-polyposis colorectal cancer (HNPCC, or Lynch-syndrome) is a hereditary autosomal dominant disease leading to early onset colorectal cancer as well as endometrial and ovarian cancer, and accounts for roughly a quarter of all MSI tumours and 3-5 % of all CRC's. It is caused by germline mutations in genes from the MMR system (MLH1, MSH2, MSH6, and PMS2), and therefore can be seen as a pure form of MSI [27, 35, 36]. The remainder of MSI tumours are called sporadic MSI tumours, and in the majority of the cases are caused by epigenetic changes, in particular methylation of the MLH1 promoter, which leads to subsequent silencing of the MLH1 gene. Absence of hypermethylation of MLH1 should prompt further analysis for Lynch syndrome. MSI colon cancers are more often located in the right-sided colon with poor differentiation and/or mucinous histology [37]. MSI tumours account for approximately 20 % of the stage I and II CRC's, 12 % of stage III and 5 % of stage IV [38]. In low stage CRC's MSI-tumours usually have better prognosis, and show response to 5-FU based chemotherapy regimens [39].

The third mechanism is characterized by *epigenetic* instability, due to aberrant hypermethylation of CpG dinucleotide sequences. These CpG islands, regions with high frequency of CpG sites, are located near to around 40-50 % of human gene promoters, whilst in adjacent mucosa these CpG islands are not methylated [25, 40]. Hypermethylation of promotors results in inappropriate transcriptional silencing of gene expression [41]. In cancer function of genes involved in cell cycle regulation, apoptosis, angiogenesis, DNA repair, invasion and adhesion is impaired [33]. CIMP-positive (CIMP+) tumours have similar clinical characteristics as MSI tumours, i.e. proximal location, mucinous histological type, higher age at diagnosis and higher occurrence in females [42]. Tumours both CIMP+ and MSI have a relatively good prognosis, however in the absence of MSI these tumours are characterised by more advanced pathology and poorer clinical outcome [43].

In addition to all three of these mechanisms, micro-RNA's are gaining interest for their post-transcriptional capability to regulate expression of onco- and suppressor genes [33, 44].

The majority of CRC's are sporadic (70-75 %), whilst up to 5 % are hereditary (i.e. Lynch-syndrome, FAP, etc.). Familial adenomatous polyposis (FAP) syndrome accounts for 1% of the new CRC patients, at an average age of 39 years, caused by a mutation in the APC gene. FAP-patients have a 100% risk of developing CRC, and once developed 5-year survival does not exceed 20 % [45]. 20-25 % of the CRC's have an increased familial risk, in whom the underlying molecular mechanism or is yet unknown, and to be identified in the future [46].

In addition to inherited or familial disease, several risk factors are known to contribute to the development of CRC. These include high (red and processed) meat and fat intake, low fruit diet, obesity, smoking and alcohol [47, 48]. Furthermore, inflammatory bowel diseases (IBD), Crohn's- and ulcerative colitis, is the third commonest condition associated with an increased risk for CRC, after FAP and HNPCC [49]. CRC's in IBD-patients account for <2 % of all CRC's, and risk of CRC's is mainly dependent of the duration of the IBD. Despite drug-treatment, IBD-patients may still develop CRC due to their chronic inflammatory state [50]. Another interesting finding pointing towards the importance of (chronic) inflammation, is the observation that long-term use of Aspirin seemed protective against the development of CRC, with reduced incidence and mortality [51].

2.3 Diagnostics

Many patients remain asymptomatic up to their diagnosis, or have aspecific symptoms [52, 53]. Therefore, a nation-wide screening programme started in the Netherlands in 2014 [54]. Evidence suggests that this biennial screening may reduce mortality with 16 % [55]. As a result of this screening, there is a significant stage-shift where relatively more low-stage cancers are diagnosed and fewer high-stage [56]. Diagnosis is proven by a pathologist after evaluation of tissue biopsies obtained by endoscopy. Alternatively, CT colonography is performed with acceptable sensitivity and specificity, however nog biopsies can be taken, nor can the site of the cancer be marked [57, 58]. X-ray or CT of the chest is used to detect potential metastasis, and MRI may be useful in selected cases, or for rectal cancer [59].

2.4 TNM Classification

The extent of tumour growth, and whether or not it has spread regionally or systemically is described by the tumour-node-metastasis (TNM) classification. This classification is used to guide treatment, and to inform the patient with regard to prognosis. Furthermore, clear classification of the tumour characteristics benefits comparison between publications worldwide. The TNM classification is based on 3 parameters, tumour depth (T), regional nodal metastasis (N) and distant metastasis (M). In the Netherlands the 8th edition of the TNM is currently implemented in the guidelines for the treatment of colon cancer [60]. **Table 1** shows a summary of this edition, including the corresponding stage grouping. Patients diagnosed with colon cancer, used to be roughly equally divided over stage I to IV in the Netherlands. However, as mentioned before, a shift of stage at presentation is seen since the implementation of the national screening program. Less than 10 % of the patients that were diagnosed by the screening program were stage IV, whilst almost 50 % of these patients were stage I. This has an impact on the prognosis and the approach of colon cancer patients, as will be discussed later in this introduction.

Table 1: TNM Staging of colon cancer (AJCC cancer staging, 8th edition)

Stage	Tumour	Node	Metastasis	Notes
0	Tis			Carcinoma in situ, intra-mucosal
I	T1-T2			Invades submucosa (T1) or muscularis propria (T2)
IIA	T3			Invades subserosa
IIB	T4a			Invades visceral peritoneum
IIC	T4b			Invades adjacent structures
IIIA	T1-T2	N1/N1c		Metastasis in 1 (N1a) / 2-3 (N1b) regional lymph nodes or deposits (N1c)
	T1	N2a		Tumour cells in 4-6 regional lymph nodes
IIIB	T3-T4a	N1/N1c		
	T2-T3	N2a		
	T1-T2	N2b		Tumour cells in 7 or more regional lymph nodes
IIIC	T4a	N2a		
	T3-T4a	N2b		
	T4b	N1-N2		
IVA	Any T	Any N	M1a	Metastasis only in 1 organ, without peritoneal deposits
IVB			M1b	Metastasis to 2 or more locations, without peritoneal deposits
IVC			M1c	Peritoneal metastasis

2.5 Treatment

2.5.1 Surgical

The treatment of colon cancer mainly depends on the stage at presentation, but surgery is the most common treatment. In 2019, 95 % of the stage I to III colon cancer patients in the Netherlands underwent surgery [61]. Reasons to refrain from surgery include metastasized disease, high age, severe comorbidities at patient's individual request, or T1 or Tis laesions after radical polypectomy [62, 63]. Technically, segmental resection with mesocolic D2 lymphadenectomy is advised. Subtotal colectomy may be chosen in order to facilitate surveillance by reducing the length of remaining colon, and to reduce the need for repetitive abdominal surgery for new malignancies, for instance in Lynch patients [64]. Excision of D3 lymph nodes (central nodes) is not yet sufficiently supported by literature [65, 66]. The minimum amount of lymph nodes that needs to be assessed by a pathologist is still subject of debate. Traditionally, a minimum of 12 lymph nodes was advised [67]. Although it is known that the amount of lymph nodes is associated with prognosis, an absolute cut-off point has not been validated [68]. In the most recent guideline in the Netherlands, 10 negative nodes are accepted as well.

2.5.2 Adjuvant chemotherapy

In general, stage III colon cancer patients require chemotherapy after surgical resection, and stage II colon cancer patients do not. However, several high risk features may lead to adjuvant treatment of stage II colon cancers. These features traditionally include T4-stage, tumour perforation, obstructive cancer, poorly differentiated histology, venous invasion or less then 10 lymph nodes harvested for pathological evaluation [69]. Recently especially T4 is recognized as a high risk feature for stage II tumours, and these tumours might benefit from adjuvant treatment, except the tumours that are microsatellite instable (MSI) [70-72]. When indicated, adjuvant treatment should start 4 to 8 weeks after surgery. High risk stage II and III patients with indication for adjuvant chemotherapy will undergo the CAPOX-regime, consisting of intravenous oxaliplatin combined with oral capecitabine. For low risk stage III patients CAPOX or FOLFOX (intravenous oxaliplatin, folinic acid and fluorouracil) is advised. Individual patient characteristics, e.g. patient's fitness, age and post-operative complications, may influence the choice of therapy. In the Netherlands, a digital

decision aid is offered to patients in several hospitals. Patients can use that aid to decide what adjuvant treatments fits their situation best, in consultation with their specialist [73].

2.6 Pathology

After surgical resection, specimens are reviewed by the pathologist. A minimal set of clinical, macroscopical and microscopical parameters is required for an adequate pathology report, as recorded in a nationwide protocol [74]. A margin of 1mm or less is considered tumour-positive. In case of positive lymph nodes, both the margin of the tumour and the margin of the lymph node are described (if that lymph node is closer to the resection margin than the tumour). Patients with primary CRC are immunohistochemically analysed for mismatch repair (MMR) proteins to exclude Lynch-syndrome and to assess MSI-status, using a panel of MLH1, PMS2, MSH2 and MSH6. MSI status can also be detected bij analysis of deficient DNA mismatch repair (dMMR) by PCR. In the future MSI-status may be increasingly reported using NGS-analysis, where mutations within microsatellite sequences of tumor samples are detected. Sometimes other molecular features are reported, like mutational status of BRAF, KRAS and NRAS.

2.7 Follow up

After initial surgical treatment, whether or not combined with adjuvant therapy, patients are followed to detect potential recurrent disease or metastases as early as possible, containing regular serum CEA monitoring, CT-scan of the chest and abdomen and colonoscopy after one year [75, 76]. The efficiency of follow-up in general is debated, since the effect on overall survival may be limited, but aim is to detected recurrent cancers in an earlier phase, at which more patients could be treated with curative intent, however at higher costs [77-79].

2.8 Survival

Overall relative 5-years survival has increased from 53 % in the early 90's to around 64% between 2010 and 2015, ranging from 95 % (stage I) to 11 % (stage IV). For stage II and III survival rates of 85 % and 68 % have been reported respectively [80]. Comparable distribution of survival over stages I to IV can be seen in international (western) registries [81].

Disease recurrence after resection of unmetastasized disease in general is seen in approximately 30 % of the patients. Thanks to the use of adjuvant chemotherapy, disease free survival rates of stage II and III patients nowadays reach up to 85-90 % and 70-75% respectively [82-84]. Note, disease free survival rates would be even lower when no adjuvant chemotherapy was used [69, 85]. However, the extent to which adjuvant chemotherapy improves survival is subject of debate [86], but may range from 10-16 % [87, 88]. In stage IV patients relapse is seen in up to 65 % of the cases [89].

Intraluminal local recurrences and metachronous cancers are found in 2-4 % of the surgically treated primary cancers. The majority of tumours detected by surveillance colonoscopy are stage I or II, and usually are treated by repeat resection with curative intent [89]. Approximately 20 % of the surgically treated colon cancer patients will develop metachronous metastases, with a median time to metastasis of 1,5 years. The majority of these metastases is found in the liver (67 %), although liver-only metastases represent 35 % of the diagnosed recurrences. Lung-only metastases represent 8 % of the recurrences in colon cancer, in contrast to the 22 % in rectal cancer, and 10 % have both liver and lung metastases. On the contrary, peritoneal metastases are seen more often in colon cancer compared to rectal cancer, 15 % and 3 % respectively [90, 91]. 16 % of the patients develop metastases at more than 2 sites.

Survival after metachronous metastases used to be poor, from 50 % at 1 year to around 5 -10% at 5 years for selected cases. However, survival of these patients have improved due to the increased use of chemotherapy, but especially due to the increase of patients who undergo surgical treatment, like hepatic metastasectomy [92]. Even patients with recurrent hepatic metastases may benefit from such (repeat) surgical treatments, with overall survival ranging from 54 to 60 months [93].

3. INTRODUCTION TO THIS THESIS

Although colon cancer is characterized by a wide range of clinical outcomes, it has a potential deadly course. Trends in incidence and survival are subject to several factors. For one, people worldwide are getting older and thus have

a higher chance of developing cancer in general. Second, colorectal cancer is considered a consequence of our western lifestyle and one of the clearest markers of epidemiological and nutritional transition. Therefore, an increase of CRC rates (and other cancers linked to western lifestyles) is seen in countries that are undergoing rapid societal and economic changes, whilst previous high rates of infection-related cancers are declining [94, 95]. Furthermore, screening programs may reduce death rates by causing a stage shift from hardly curable stage IV to better curable localized disease. This shift to localized disease means an increase of patients where there continues to be a debate on optimal post-operative risk assessment and management, i.e. whether or not adjuvant chemotherapy is administered [96].

For now, stage III patients and high risk stage II patients are treated with adjuvant chemotherapy, although the routine-use of chemotherapy even in stage III is sometimes questioned [92]. For stage II several prognostic parameters are used, besides some pathological and clinical markers like higher T4, suboptimal lymph node retrieval, presence of lymphovascular invasion, bowel obstruction or bowel perforation, and poorly differentiated histology [97]. However, T4 seems the only consistent poor prognostic parameter [98, 99]. Furthermore, microsatellite instability (MSI) is closely linked to improved outcome and to limited benefit of adjuvant fluoropyrimidine therapy [100, 101]. Therefore, stage II MSI-patients are not adjuvantly treated. MSI-status is the only marker currently used routinely in non-metastasized colon cancers.

For metastasized cancers KRAS, as a downstream effector of the epidermal growth factor receptor (EGFR), has some prognostic and predictive value [102, 103]. Furthermore, a mutation in the BRAF-gene (V600E), is associated not only with reduced overall survival, but also DNA mismatch repair status, although its clinical value needs further elucidation.

In order to further improve identification of high-risk subgroups several commercial prognostic gene signatures have been developed, like ColoPrint and OncotypeDX [104, 105]. However, these signatures face practical implementational hurdles and are expensive, whereas easily available factors such as T4 and MSI-status appear as stronger markers [103, 106].

In a further attempt to elucidate the biological diversity of colon cancers, several gene-expression based classifications have been published. These studies aimed to describe the relation between biological and clinical characteristics and to identify poor-prognosis subtypes or stratify subtypes for their response to adjuvant or targeted treatment [107-114]. Ultimately, these study groups formed an international consortium to resolve discrepancies between their classifier and to collaborate on bioinformatics, resulting in the largest collection of CRC cohorts [115]. This resulted in the consensus molecular subtypes of colorectal cancer, consisting of four types of colon cancer with distinguishing features, CMS1 to CMS4. CMS1 tumours (representing 14 % of the tumours) are microsatellite unstable, hypermutated and CIMP-high tumours. This subgroup shows enrichment of BRAF mutations and immune infiltration, and clinically is characterized by poor survival after relapse. CMS2 (37 %) are the chromosomal unstable tumours with activated β-catenin-dependent Wnt-signalling. CMS3 (13%) are the tumours characterized by metabolic dysregulation that present as CIMP-low with mixed MSI-status, enriched for KRAS mutations. CMS4 (23%) are the mesenchymal-like tumours with a stromal infiltration and angiogenesis profile, that have poor relapse-free and overall survival. The remaining 13 % may represent a transition phenotype, or is based on intra-tumoural heterogeneity [115]. Although this classifier might be a robust classification system, that can stratify a CRC patient according to a biological profile, clinical implication still remains difficult. Just as most markers, this classifier may provide us information of biological behaviour and overall prognosis on a group level. However, accurate selection of a specified patient with upcoming recurrence, or that tumour that will not recur, still remains challenging.

Recurrence rates as they are now for both stage II and III offer room for improvement. 5-years overall survival for surgically treated stage II colon cancer patients is estimated around 80 % with limited survival benefit after adjuvant treatment (3,6%) [96, 116]. This means that around 20% of the stage II patients may develop recurrence, or in other words, have residual micro metastatic disease. Some of these patients may respond to adjuvant treatment, but some may not [117]. Even in stage III, where adjuvant treatment is routinely advised, there might be a proportion of patients receiving chemotherapy, and its side effects, but would not have developed disease recurrence. On the other hand, some patients would have developed recurrence anyway, regardless of adjuvant

chemotherapy, but also do suffer the side effects. So the heterogeneity of the population, even within each stage, may hamper adequate risk stratification with current methods.

4. AIM AND OUTLINE OF THIS THESIS

In the future we will more frequently have to answer the questions: is this patient cured after surgery or not? Or, who has residual disease and/or will develop recurrence, and who does not. As we learn more and more about the underlying biology, biomarkers will play a prominent role in this process. Therefore, the general aim of this thesis is to contribute to this quest for prognostic biomarkers and to improve the identification of stage II and III sporadic colon cancer patients at risk for disease recurrence.

In **Chapter 2** we describe the prognostic value of microvessel density (MVD) as a derived marker for angiogenesis, one of the hallmarks of cancer. This study describes tumour morphology, rather than biomarker analysis. A digital image processing program was used on whole tissue sections of 107 stage II and III colon cancer patients, to identify the CD31-stained endothelial lining of blood vessels and to calculate the density of blood vessels within the tumour. After this morphological study, we proceed to more biomarker driven studies, starting with validation of one of the most promising biomarkers at that time, CDX2. In **Chapter 3** we aimed to analyse and validate the prognostic value of CDX2 using routine immunohistochemistry on tissue micro arrays (TMA). In addition, CDX2 expression was analysed by tandem mass spectrometry as a more quantitative measure for expression of this marker. While validation of a promising immunohistochemical marker on other (well-known) cohorts is valuable itself, this study also shows that validation, and ultimately implementation, of biomarkers may be laborious. Interestingly, even though a biomarker cannot be validated immunohistochemically, clinical value of that marker may be proven otherwise, by alternative (or more quantative) techniques. Whilst analysis of CDX2 was performed on whole tumour tissue, both stroma and epithelium, the next study focusses on biomarker expression specifically in the epithelium of colon cancer tissues. KCNQ1 and its prognostic value was analysed and presented in **Chapter 4** as a new potential biomarker, previously showing promising results

in mice. Immunohistochemical and mRNA analysis are described, in addition to expression analysis of CD44, both being markers activated by Wnt-signalling. Immunohistochemical expression of KCNQ1 and CD44 was examined in TMA's of 386 stage II and III colon cancer patients, and mRNA expression of KCNQ1 in an external cohort of 90 patients. In **Chapter 5** we will subsequently focus on MACROD2, a gene with a fairly unknown function but known for its frequent focal DNA copy number losses. MACROD2 protein expression was investigated by TMA analysis for its prognostic value, however potential predictive value in certain subgroups was taken into account as well. In **Chapter 6** we describe a final attempt to more accurately predict prognosis, by combining several immunohistochemical markers into a clinically applicable classifier. We therefore performed a Classification and Regression Tree (CART) analysis on all markers evaluated and published previously in our cohort, to extract the best possible classifier for stage II and III colon cancer patients. **Chapter 7** and **8** contain the summary, discussion, future perspectives and the dutch summary of this thesis."

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CHAPTER 2

Prognostic value of microvessel density in stage II and III colon cancer patients: a retrospective cohort study

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ABSTRACT

Background

Microvessel density (MVD), as a derived marker for angiogenesis, has been associated with poor outcome in several types of cancer. This study aimed to evaluate the prognostic value of MVD in stage II and III colon cancer and its relation to tumour-stroma-percentage (TSP) and expression of HIF1A and VEGFA.

Methods

Formalin-fixed paraffin-embedded (FFPE) colon cancer tissues were collected from 53 stage II and 54 (5-fluorouracil-treated) stage III patients. MVD was scored by digital morphometric analysis of CD31-stained whole tumour sections. TSP was scored using haematoxylin-eosin stained slides. Protein expression of HIF1A and VEGFA was determined by immunohistochemical evaluation of tissue microarrays.

Results

Median MVD was higher in stage III compared to stage II colon cancers (11.1% versus 5.6% CD31-positive tissue area, $p < 0.001$). High MVD in stage II patients tended to be associated with poor disease free survival (DFS) in univariate analysis ($p = 0.056$). In contrast, high MVD in 5FU-treated stage III patients was associated with better DFS ($p = 0.006$). Prognostic value for MVD was observed in multivariate analyses for both cancer stages.

Conclusions

MVD is an independent prognostic factor associated with poor DFS in stage II colon cancer patients, and with better DFS in stage III colon cancer patients treated with adjuvant chemotherapy.

INTRODUCTION

The physiological role of angiogenesis at adult age is confined to wound- and bone healing and the female reproductive cycle, and therefore is activated temporarily. In contrast, the angiogenic pathway is often constitutively activated in tumours to meet their needs for nutrients and to facilitate tumour growth and metastasis. This is an early event in the development of cancer and can already be observed in pre-malignant lesions [1-3]. However, these newly formed blood vessels have a less distinct organized hierarchy [4], and are prone to vascular leakage for their irregular endothelial layer with intermediate spaces [5]. Interstitial blood pressure is increased leading to compromised blood flow and thus hypoxia and acidosis [6,7]. Expression of the alpha subunit of hypoxia-inducible factor 1 (HIF1A) is stabilized as a result of this hypoxia and evokes angiogenesis by the upregulation of expression of vascular endothelial growth factor A (VEGFA) [8,9]. This process is enhanced by the influence of for instance tumour associated macrophages, cancer-associated fibroblasts, and the extracellular matrix [10]. Constitutive upregulation of HIF1A and VEGFA expression can also be induced by oncogene signalling, e.g. by transforming growth factor-β (TFG-β), the involvement of the Wnt/β-catenin pathway and inhibition by the p53 pathway [11-16]. HIF1a is furthermore known to interact with apoptotic markers like Bax and Bcl-xL [17]. It leads to enhanced proliferation, survival and migration of endothelial cells, increased vascular permeability and altered gene expression [14,18,19].

Desmoplastic tumour stroma interacts with and supports the tumour parenchyma, forming a microenvironment in which the tumour can progress. The tumour stroma and the microenvironment promote angiogenesis and tumour progression and eventually metastasis [10,20]. There is increasing evidence that the proportion of this stroma in colon cancer is inversely related to survival [21,22]. Consequently, tumours with high tumour-stroma percentage (TSP) are likely to express more angiogenic factors, leading to more angiogenesis, and angiogenesis-rich tumours may be associated with a worse prognosis.

In contrast to stroma percentage, there is no direct measure or single marker for angiogenesis to which survival can be correlated [21]. Microvessel density (MVD) has been analysed since the early 90's as an angiogenesis-derived marker

[23,24]. The hypothesis that high-MVD tumours are associated with poor prognosis was indeed proven in breast cancer, where higher MVD was associated with poor survival [25,26], and in non-small cell lung cancer where high MVD was associated with poor survival after surgery [27]. In colorectal cancer (CRC), prognostic value of MVD has remained inconclusive, although some publications suggest associations with survival [28-33].

The aim of this study was to examine the relation of MVD to disease-recurrence, in both stage II and stage III colon cancer patients, while taking into account the amount of tumour stroma (TSP) and expression of HIF1A and VEGFA.

MATERIALS & METHODS

Study design and population

Based on a previously established well-documented retrospective cohort of 386 stage II and III colon cancer patients with no prior history of CRC [34], we here selected a subset of 53 stage II and 54 stage III colon cancer patients of whom whole tissue sections were available for MVD analysis. In this subset all stage III patients were treated with adjuvant 5-FU based chemotherapy, whilst all stage II patients were treated with surgical resection only. The tumours from these patients were microsatellite stable (MSS), as previously determined by PCR analysis [34]. Clinical data and tumour tissue was obtained conform the "Code for Proper Secondary Use of Human Tissue in The Netherlands" [35]. Baseline characteristics and clinicopathological data are shown in **Supplementary Table 1** (appendices, chapter 9).

CD31 immunohistochemistry and microvessel density analysis

4 µm FFPE whole tissue sections were mounted on glass slides, deparaffinised and rehydrated. To identify (micro) vessels, sections were stained with a mouse monoclonal antibody directed against CD31 (anti human CD31, clone JC70A, catalogue number M0823, Dako, Heverlee, Belgium) in a 1/50 solution and using a Tris (pH 9) buffer for maximum retrieval in a microwave for 1 hour. A Powervision+ method (Immunologic, Duiven, The Netherlands) was used as secondary antigens, after one hour incubation at room temperature. These sections were digitized using a Mirax slide scanner system equipped with a 20x

objective with a numerical aperture of 0,75 (3DHISTECH, Budapest, Hungary) and a Sony DFW-X710 Fire Wire 1/3" type progressive SCAN IT CCD (pixel size 4,65 x 4,65 μm^2) resulting in an actual scan resolution at 20x of 0,23 µm. Monitors used for selection and scoring were calibrated using Spyder2PRO software (v.1.0-16; Panone Colorvision, Regensdorf, Switzerland). Representative tumour tissue was delineated using Pannoramic Viewer (v 1.15.3, 3DHISTECH Ltd), and damaged parts and/or absence of tissue in delineated tumours were annotated as well, and later digitally excluded for analysis. A pathologist (HB) approved the delineating process. The delineated areas were exported as high resolution TIFF-files. This resulted in TIFF files of 60 to 320 Mb with a minimum resolution of approximately 2500x3100 pixels, depending on the original tumour size. A Java-based image processing program, ImageJ (v1.47, Wayne Rasband, 64bits), was used to import the TIFF files and to perform morphometric image analysis to detect and measure the microvessels. This full script, is a CD31-specific version of previously published work [36], see **Supplementary script, Additional file 1** (appendices, chapter 9). In brief, this script analyses all stained pixels in included tissue in the delineated TIFF-files. Based on RGB colour codes, the CD31 positive cells were identified. The brown (clustered) pixels represented endothelial cells, whereas the non-brown pixel represented normal stroma, epithelial cells *etc*. A size-threshold of minimal hundred CD31-positive pixels was used as minimal size of microvessels. The total percentage of CD31-positive (clustered) pixels per tissue area analysed is used as measure for the average microvessel density in that whole tumour section.

Tumour-stroma percentage

For TSP analysis, 4 µm FFPE tissue sections were mounted on glass slides, deparaffinised and rehydrated, and stained with haematoxylin-eosin (HE). Neoplastic epithelium and stroma was quantified using QProdit (Leica) stereology software. The borders of the tumour in each section were annotated. Subsequently, the software generated a 400-point grid for a 20x objective within these borders. At each point, the tissue was scored for epithelium or stroma. Tumour-stroma percentage was calculated using the number of stromal hits divided by the total number of both epithelial and stromal hits. Since some sections were damaged, 99 patients remained for analysis.

HIF1A and VEGFA immunohistochemistry and TMA analysis

Tissue micro arrays (TMA) were generated from these patients as described previously [37]. Expression of HIF1A and VEGFA was determined by immunohistochemical evaluation of TMAs, using previously established workflows [37]. For HIF1A, antigen retrieval was performed in antigen retrieval solution (DAKO, Glostrup, Denmark) for 45 minutes at 96 ºC. Then, the primary antibody against HIF1A (mouse monoclonal, clone 54, catalogue number 610958, BD, Franklin Lakes, USA) was incubated for 30 minutes with a 1/500 dilution at room temperature. The amplification reagent from the Catalazyd Signal Amplification system (CSA, Dako kit) was used for detection of the staining. For VEGFA expression (mouse monoclonal, clone VG1, catalogue number MS-1467-P1, Neomarkers, Fermont, USA) a Tris (pH 9) buffer in the microwave was used for antigen retrieval, with subsequent incubation for one hour and secondary visualization also by a Powervision+ method.

Protein expression analysis of TMAs was performed as described previously [37]. In brief, six cores per patient were examined and scored blindly for intensity (negative, weak, moderate and strong) of stained cells, using dedicated TMA scoring software (Pannoramic Viewer, v1.15.5; 3DHISTECH Ltd). Scores were internally corrected for stromal- and background staining. Damaged and missing cores were not scored. Expression was scored in cytoplasm of epithelial cells. Scores were obtained for 103 (HIF1A) and 100 (VEGFA) patients, respectively. For further statistical analyses, all scores were converted to dichotomous values using ROC-based cross-validation analysis [37].

Statistical analysis

Kolmogorov-Smirnov (KS) test was used to assess the normality of the distribution of MVD- and TSP-values. For analysis of differences in clinical and histological baseline parameters between study groups (stage and MVD) independent-t-testing, Mann-Whitney U and chi-square tests were used. In both stage II and III, continuous MVD- and stromal data was dichotomized for further (survival) analysis, based on highest specificity and sensitivity in ROC-analysis. This resulted in high-MVD when MVD was higher than 5.45% for stage II, and higher than 8.91% for stage III colon cancer. Stroma was subsequently defined as high if TSP > 43,1% for stage II, and TSP > 49,2% for stage III colon cancer. Difference in disease free survival (DFS) was visualised with Kaplan

Meier curves and log-rank. Hazard ratio's (HR) and 95% confidence interval (95% CI) were estimated with cox-regression analysis. Multivariate analysis was performed using stepwise backwards Cox regression, with DFS as dependent variable (p-out = 0.1). Similar statistics were performed on expression scores of HIF1A and VEGFA. Associations between MVD, TSP, HIF1A and VEGFA were analysed with chi-square tests and spearman's rho-test. All statistical analyses were processed in SPSS (IBM SPSS Statistics for windows, SPSS Inc., Chicago, Illinois, USA), with two-sided analysis and a significance level of p< 0.05.

RESULTS

MVD in stage II and stage III colon cancer

For determining MVD, whole tissue sections were stained with CD31 (**Figure 1a).** Baseline clinical and pathological data characteristics of the cohort are presented in **Supplementary Table 1, Additional File 2** (appendices, chapter 9), stratified for stage II and III colon cancer. Besides differences that are inherent to a comparison of stage II to stage III patients (adjuvant chemotherapy, T- and N-stadium), stage III patients were significantly younger (65,5 versus 72.7 years; p=0.004) and had significantly more angioinvasion, defined by the observation of epithelial cells within the (lymph)vascular lumen (38.9 % versus 11.3 %; p=0.001).

Mean MVD of 107 tumours was 10.4 %, with a median of 9.0. In stage III tumours, MVD was significantly higher compared to stage II (11.1% and 5.6 %, respectively; p<0.001, **Figure 2a).** Within stage II, no differences were seen in clinicopathological characteristics between MVD-high and –low group. In stage III tumours, only more ulceration (p=0.042) and less recurrences in high-MVD patients (p=0.013) were observed. Clinicopathological characteristics for high *versus* low MVD, within both stages, are presented in **Table 1.**

Table 1: Baseline characteristics and clinicopathological data stratified for low and high MVD for both stage II and III colon cancer. P-values were calculated by chi-square test or independent t-testing for continuous data. Significant p-values are printed in bold.

Clinicopathological characteristics	**Stage II: n= 53 (%)**			**Stage III: n= 54 (%)**		
	MVD-low n= 26 (%)	**MVD-high n= 27 (%)**	**p-value**	**MVD-low n= 15 (%)**	**MVD-high n= 39 (%)**	**p-value**
Sex						
Male	14 (53.8)	14 (51.8)		10 (66.7)	25 (64.1)	
Female	12 (46.2)	13 (48.2)	0.88	5 (33.3)	14 (35.9)	0.86
Age, mean (s.d.) (years)	73.7 (11.3)	70.9 (13.3)	0.41	63.6 (9.8)	66.6 (10.1)	0.32
Right sided tumour	9 (34.6)	8 (29.6)	0.70	7 (46.7)	18 (46.2)	0.97
Diameter, mean (s.d.) (mm)	39.2 (20.7)	40.80 (19.3)	0.61	36.4 (10.5)	33.9 (13.4)	0.44
Histological grade						
Good	2 (7.7)	3 (11.1)		1 (6.7)	1 (2.6)	
Average	23 (88.5)	23 (85.2)		12 (80.0)	35 (89.7)	
Poor	1 (3.8)	1 (3.7)	0.91	2 (13.3)	3 (7.7)	0.61
Tumour stage						
T1	-	-		-	1 (2.6)	
T2	-	-		1 (6.7)	6 (15.4)	
T3	23 (88.5)	26 (96.3)		10 (66.7)	30 (76.9)	
T4	3 (11.5)	1 (3.7)	0.28	4 (26.7)	2 (5.1)	0.13
Nodal stage						
N1	-	-		12 (80.0)	22 (56.4)	
N2	-	-	-	3 (20.0)	17 (43.6)	0.11
Mucinous differentiation.	8 (30.8)	4 (14.8)	0.17	1 (6.7)	4 (10.3)	0.68
Ulceration	18 (69.2)	23 (85.2)	0.17	10 (66.7)	35 (89.7)	**0.042**
Angioinvasion	2 (7.7)	4 (14.8)	0.41	7 (46.7)	14 (35.9)	0.47
Perforation						
No	24 (92.3)	24 (88.9)		14 (93.3)	37 (94.9)	
Before surgery	1 (3.8)	1 (3.7)		1 (6.7)	1 (2.6)	
During surgery	-	1 (3.7)		-	-	
After surgery	1 (3.8)	1 (3.7)	0.81	-	1 (2.6)	0.64
Tumour spill	1 (3.8)	2 (7.4)	0.58	1 (6.7)	-	0.10
Adjuvant chemo	0	0	-	15 (100.0)	39 (100.0)	-
Recurrence	6 (23.1)	12 (44.4)	0.10	11 (73.3)	14 (35.9)	**0.013**
CRC mortality	6 (23.1)	9 (33.3)	0.41	8 (53.3)	12 (30.8)	0.12
Overall mortality	14 (53.8)	16 (59.3)	0.69	9 (60.0)	17 (43.6)	0.28
Follow up, mean (s.d.) months	70.5 (32.6)	58.1 (35.9)	0.19	52.3 (33.7)	57.7 (27.4)	0.54

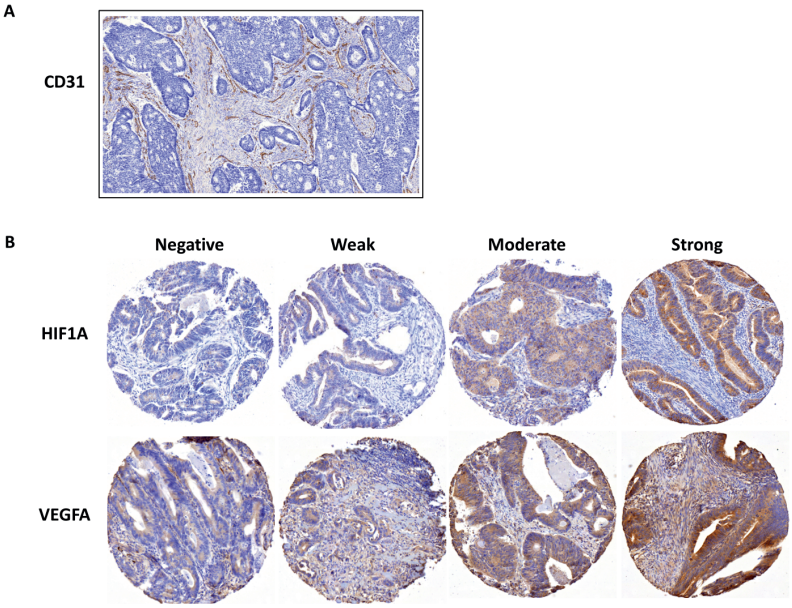

Figure 1: Examples of immunohistochemical stainings for A: CD31 on whole tissue section; and B: HIF1A and VEGFA on TMA cores, scored as negative, weak, moderate, strong.

High MVD is associated with poor DFS in stage II, and better DFS in adjuvant treated stage III colon cancers

To investigate the univariate association of MVD with survival in stage II patients, DFS was analysed and visualized using Kaplan Meier curves. For stage II, high MVD tended to be associated with worse DFS (HR = 2.53 [95% CI: 0.95-6.76]; Log-rank p=0.056, **Figure 3a).** Similarly, the univariate effect of MVD on DFS in stage III was investigated. In contrast to the association in stage II, high MVD is associated with better DFS in stage III patients, all treated with adjuvant chemotherapy (HR = 0.34 [95% CI: 0.16-0.76]; Log-rank p=0.006, **Figure 3b).**

No association between DFS and TSP, HIF1A or VEGFA

Examples of expression of HIF1A and VEGFA are shown in **Figure 1b,** and results and survival curves for TSP, HIF1A and VEGFA are presented in **Supplementary Figures 1-3, Additional File 3.** In contrast to MVD, TSP was not significantly different between stage II and III colon cancers (p=0.062, **Figure 2b**). More stroma showed a trend of being related to poor DFS in stage II (HR = 2.07 [95% CI: 0.76-

5.60]; Log-rank p=0.144, **Supplementary Figure 1a, Additional File 3).** Also for expression of HIF1A and VEGFA, no statistically significant associations were observed **(Supplementary Figure 2, 3, Additional File 3).** Supplementary figures are shown in the appendices, chapter 9.

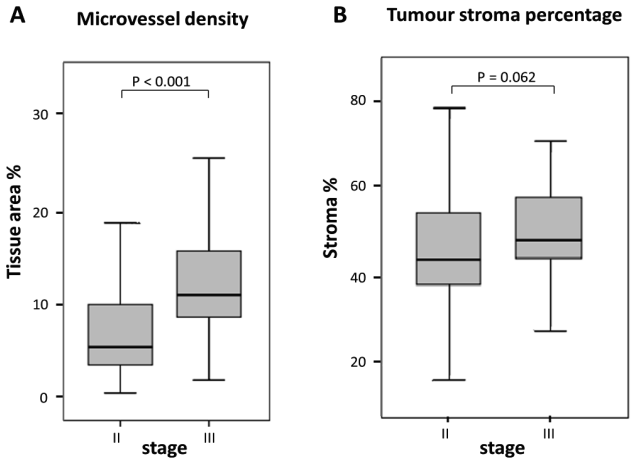

Figure 2: Comparison between stage II and stage III colon cancer patients for (A) microvessel density; and (B) tumour-stroma percentage. P values were obtained by Mann-Whitney U analysis.

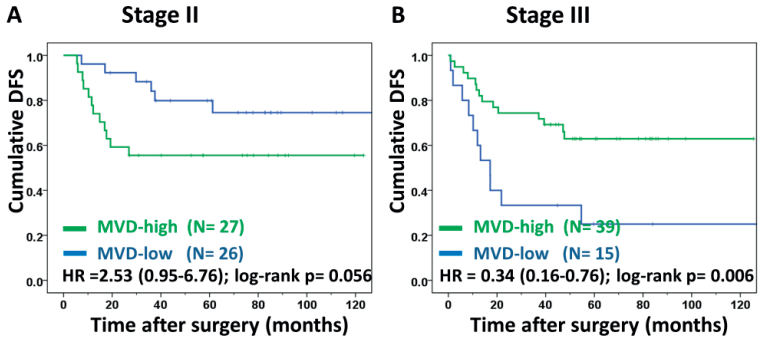

Figure 3: Kaplan Meier curves for DFS of stage II (A) and stage III (B) colon cancer patients, stratified for high and low MVD. Hazard ratio (HR), 95% confidence interval, and log-rank p-values are reported.

Microvessel density is an independent prognostic factor in stage II and III colon cancer patients

A multivariate model for 5-year DFS was built using stepwise backward Cox-regression. MVD, TSP, HIF1A, VEGFA, right-sided, diameter, degree of differentiation, ulceration and angioinvasion were included for analysis. In both stage II and stage III colon cancer, MVD was retained in the model significantly, demonstrating its added value as a prognostic biomarker in a multivariate setting.

In addition to MVD, only right-sidedness and ulceration were retained in stage II colon cancer. For stage III colon cancer ulceration and angioinvasion in stage III were retained in the model **(Table 2).** TSP, HIF1A and VEGFA were not associated with DFS in this multivariate assay. No correlations between MVD and TSP or expression of HIF1A and VEGFA were found. MVD was correlated to expression of VEGFA in stage II colon cancer (correlation coefficient -0.331, p=0.020, **Supplementary Table 2, Additional File 2** (appendices, chapter 9)).

Table 2: Multivariate backward Cox-regression analysis for 5-year disease free survival of high microvessel density and clinicopathological parameters that were retained in the model, in stage II and III colon cancer patients. HR: Hazard ratio; 95%-CI: 95% confidence interval.

Stage	Parameters	HR	95%-CI	P-value
II	Right-sided tumour	0.18	0.04-0.83	0.027
	Ulceration	6.80	0.89-52.23	0.065
	High microvessel density	**4.50**	**1.38-14.64**	**0.013**
III	Ulceration	0.32	0.10-1.06	0.062
	Angioinvasion	4.38	1.71-11.23	0.002
	High microvessel density	**0.34**	**0.13-0.90**	**0.031**

DISCUSSION

This study addressed the analysis of microvessel density and its relation to disease stage and prognosis, in microsatellite stable stage II and III colon cancer patients. MVD was higher in stage III compared to stage II colon cancers. Previously it has been shown that MVD increases during the progression from normal mucosa, through adenomas to carcinomas [38]. It is plausible that during the evolution from stage I carcinoma to metastasized disease (stage IV), angiogen-

esis and MVD are enhanced to meet the increasing demands of tumour growth and progression.

In stage II, high MVD was related to worse disease outcome, i.e. worse DFS, in particular observed as a significant effect in the multivariate analysis. Thus, stage II colon cancers with high MVD might represent a biological subset of cancers with unfavourable characteristics and a tendency to progression, in line with the observation mentioned previously, leading to worse prognosis. A similar trend, albeit not reaching statistical significance, was found for TSP. All stage III tissues were obtained from patients who were treated with adjuvant chemotherapy after resection of their primary tumour. In contrast to stage II, high MVD was related to improved DFS in stage III patients. Stage III cancers already proved to have lymphatic potential, and can only progress to metastasized disease, stage IV. Although the primary tumours are already resected, residual tumour tissue, whether located in lymphatic tissue or already as subclinical distant metastasis, potentially exert the same tumour characteristics as the primary tumour [39]. Therefore, residual tumour tissue or early recurrent tumours, from primary tumours with high MVD, might have higher MVD as well and potentially allow better penetration for the adjuvant 5-FU based chemotherapy. This might explain the better prognosis of 5-FU treated stage III colon cancers with high MVD. It seems that even though high-MVD stage III cancers should have worse prognosis when untreated, they actually might predict better response to adjuvant chemotherapy. This hypothesis might also explain why Bevacizumab in stage II colon cancer does not improve DFS [40]. In stage II, with already fewer (micro)vessels present, only patients with high MVD (high risk) might benefit from inhibiting formation of new vessels. This might explain why there was no improvement of DFS for the entire group of stage II patients, since the group with favourable prognosis (low MVD) might show no further improvement by reduction of the already low vessel density. Potentially there is benefit from Bevacizumab in lower stages of colon cancer, though restricted to selected cases with high MVD.

Tumour-stroma percentage and the expression of HIF1A and VEGFA, although functionally interconnected, were not significantly associated with MVD in this study, except for correlation between MVD and expression of VEGFA in stage II.

Interestingly, for the association of stromal percentage with DFS for stage II and III, an opposite effect on DFS was observed in both stages similar as for MVD.

With regards to the method of MVD-analysis, several measures were taken to avoid some well-known methodological difficulties. In literature concerning MVD in colon cancer, results on prognosis were ambiguous, possibly for its wide range of methods. Antibodies used to visualize endothelial cells differ amongst studies (CD31, CD34, factor VIII, miRNA-126) [28], and sampling of the measurement area within the tumour is another critical factor. It is accepted to define MVD in 'hot-spots', but there is no consensus about the number of hotspots needed to count [24,29-31,41]. Furthermore, 'hotspot' may refer to the invasive margin of the tumour, or the area in the tumour with highest MVD by 'eyeballing'. Both selection methods may be observer-dependent. Finally, microvessels can be counted manually or digitized using quantitative image analysis, of which the latter has proven to have more accuracy and prognostic relevance [24]. These and other ambiguities may contribute to the fact that MVD is not unanimously described as a prognostic factor, prohibiting it from being implemented in standard histopathological examination. To avoid such observer-dependent area selection, MVD was analysed digitally and in whole sections in which the entire tumour-area was annotated. This excluded both the bias of hot-spot diameter/selection, as the disadvantages of manual counting. It contributed to a more robust, feasible, reproducible and observer-independent method. To identify endothelial cells, CD31 antibodies were used as a commonly accepted marker [27], taking into account that it can be found on platelets and white blood cells to some degree as well. On the other hand, it is more sensitive for younger and more immature vessels.

CONCLUSIONS

MVD is a surrogate marker of angiogenesis in tumours, direct measurement of which so far has remained impossible. Still, measuring MVD remains subject to some practical challenges, of which some were tackled in this study. In the present study, an increased MVD was seen in stage III colon cancer patients, in comparison to stage II. MVD appeared to be an independent prognostic factor associated with poor DFS in stage II colon cancer patients, and with better DFS

in stage III colon cancer patients who were treated with adjuvant 5-FU based chemotherapy afterwards. This latter observation may be of particular clinical interest, pending further validation.

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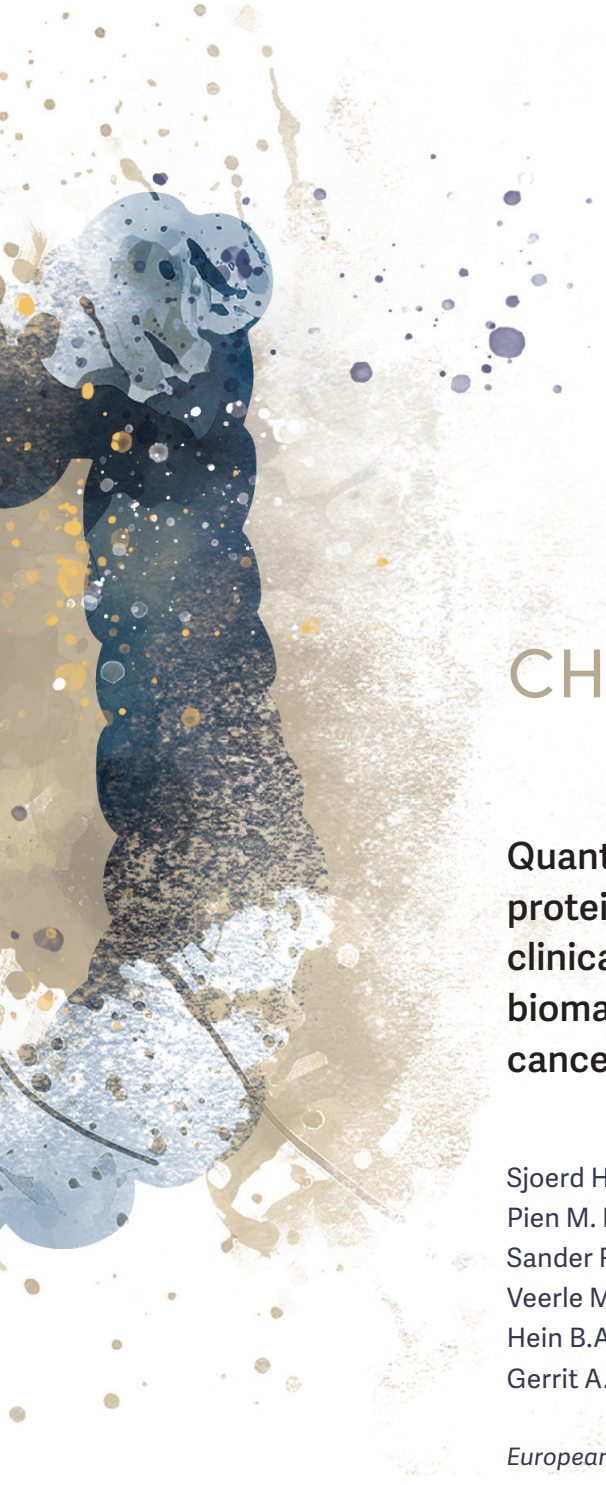

CHAPTER 3

Quantitative analysis of CDX2 protein expression improves its clinical utility as a prognostic biomarker in stage II and III colon cancer

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ABSTRACT

Aim

Better stratification of patients with stage II and stage III colon cancer for risk of recurrence is urgently needed. The present study aimed to validate the prognostic value of CDX2 protein expression in colon cancer tissue by routine immunohistochemistry and to evaluate its performance in a head-to-head comparison with tandem mass spectrometry ebased proteomics.

Patient and methods

CDX2 protein expression was evaluated in 386 stage II and III primary colon cancers by immunohistochemical staining of tissue microarrays and by liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis using formalin-fixed paraffin-embedded tissue sections of a matched subset of 23 recurrent and 23 non-recurrent colon cancers. Association between CDX2 expression and disease-specific survival (DSS) was investigated.

Results

Low levels of CDX2 protein expression in stage II and III colon cancer as determined by immunohistochemistry was associated with poor DSS (hazard ratio (HR) = 1.97 [95% confidence interval (CI): 1.26-3.06]; p=0.002). Based on analysis of a selected sample subset, CDX2 prognostic value was more pronounced when detected by LC-MS/MS (HR = 7.56 [95% CI: 2.49-22.95]; p < 0.001) compared to detection by immunohistochemistry (HR = 1.60 [95% CI: 0.61-4.22]; p=0.34).

Conclusion

This study validated CDX2 protein expression as a prognostic biomarker in stage II and III colon cancer, conform previous publications. CDX2 prognostic value appeared to be underestimated when detected by routine immunohistochemistry, probably due to the semiquantitative and subjective nature of this methodology. Quantitative analysis of CDX2 substantially improved its clinical utility as a prognostic biomarker. Therefore, development of routinely applicable quantitative assays for CDX2 expression is needed to facilitate its clinical implementation.

INTRODUCTION

Colon cancer ranks third on incidence in Europe with almost 500,000 new patients and over 240,000 deaths in 2018 [1]. In patients with stage II and III colon cancer, there is an urgent clinical need to identify those patients who would (not) benefit from adjuvant chemotherapy (ACT). At present, patients with stage II tumours only have an indication for ACT when high-risk features are present. These features include T4, obstruction and/or perforation, vascular invasion or resections in which the recommended number of lymph nodes to be analysed cannot be reached [2-4]. With these clinical guidelines as standard of care, still around 12e15% of stage II patients develop recurrent disease [5,6]. Patients with stage III cancers who are treated with ACT still develop recurrences in 24e33% of cases [5,6]. There is also a large subgroup of stage III patients who would have been cured by surgery alone, receiving futile ACT in current treatment regimens [7]. Taken together, diseasefree survival (DFS) increased from 55% to 66% upon treatment with ACT [8-10], indicating that approximately only 11% of patients with stage III colon cancer are likely to benefit from ACT, whereas all others unnecessarily suffer from its side effects. As routine pathological approaches are currently insufficient to accurately estimate the risk of recurrence for patients with stage II or III colon cancer, a more accurate stratification of an individual patient's risk for disease recurrence is needed to better decide who to offer additional treatment after surgery.

One biomarker with prognostic value that has the potential to be implemented in a routine clinical diagnostic setting is protein expression of caudal-related homeobox transcription factor 2, or CDX2. Immunohistochemical staining of CDX2 is already part of the repertoire of many pathology labs, where it is mainly used to classify adenocarcinoma metastases by likely site of origin, where CDX2 expression points towards the colon [11].

The CDX2 protein is expressed in the nuclei of intestinal epithelial cells. Its function is related to Wnt-signalling, homeostasis and permeability, and it is involved in regulation of multiple genes expressed in the intestinal epithelium [12-16]. Therefore, it is part of several important functional pathways, ranging from early differentiation to maintenance of the intestinal epithelial lining. In cancers, homeobox genes in general are often deregulated [15,17]. Knockdown

of CDX2 in colon cancer cells is known to promote cell proliferation in vitro and accelerates tumour formation in vivo [16], whereas overexpression of CDX2 was shown to inhibit cell proliferation. Furthermore, CDX2 has been used as a keymarker to distinguish between the mesenchymal-like and epithelial-like consensus molecular subtypes (CMS) [18,19].

Importantly, loss of CDX2 expression was associated with poor survival [20-23]. Nevertheless, these findings have not yet resulted in clinical implementation of

CDX2 as a prognostic biomarker, mainly due to lack of validation studies, reliable and robust assays, costeffectiveness studies and biomarker-driven clinical trials. The present study aimed to validate CDX2 as a prognostic biomarker in a single centre well-defined cohort of patients with stage II and III colon cancer. The second aim was to examine the impact of the methodology used for detecting CDX2 protein expression. To this end, the routinely used semiquantitative immunohistochemical evaluation (categorical data) was compared to a quantitative approach, i.e. tandem mass spectrometryebased proteomics (continuous data).

PATIENTS AND METHODS

Study design and population

The study population comprised 386 patients with sporadic colon cancer, of whom 226 were stage II and 160 stage III. All patients were treated in the Spaarne Gasthuis (formerly Kennemer Gasthuis) hospital in the Netherlands and were included retrospectively [24]. Collection, storage and use of clinicopathological data and tissue specimens were performed in compliance with the 'Code for Proper Secondary Use of Human Tissue in The Netherlands', conform local and national legislation that was applicable at the time, as described previously [24,25]. Microsatellite instability (MSI) status was previously determined and available for 296 of 346 patients [26]. End-points for survival were DFS, defined as time from surgery to recurrent disease, and diseasespecific survival (DSS) defined as time from surgery to cancer related death. Median follow up was 57.1 months.

CDX2 immunohistochemistry

Tissue microarray (TMA) recipient blocks with 6 cores from each patient were obtained from annotated tumour tissues from formalin-fixed paraffin-embedded (FFPE) donor blocks [27]. All TMAs were immunohistochemically stained with CDX2 rabbit monoclonal primary antibody (clone EPR2764Y, Cell Marque, Rocklin, USA) using a BenchMark Ultra autostainer (Ventana Medical Systems, Oro Valley, USA). More details are described in supplementary methods, SM1 (appendices, chapter 9). TMAs were scored by a pathologist for intensity (categorised into negative, weak, moderate and strong) as described previously [27]. For 40 patients scoring of CDX2 expression was not possible due to missing cores or core damage, i.e. more than 2/3 of the core surface lacking or folded, leaving 346 of 386 patients for evaluation. CDX2 expression was scored in the nucleus of epithelial cells. The tissue core with the highest score per patient was used for further statistical analyses and converted to dichotomous values using receiver operating characteristic (ROC)-based cross-validation analysis [27]. Based on this analysis, 'moderate' and 'strong' were 'CDX2- high', whereas 'negative' and 'weak' were 'CDX2-low'.

CDX2 FFPE tandem mass spectrometry proteomics

The prognostic value of CDX2 was subsequently analysed using a more quantitative method yielding numerical data, in an exploratory analysis. From the 386 stage patients, 23 patients with disease recurrence were randomly selected and matched to 23 non-recurrent patients, based on a nested case-control design. Matching was based on age, disease stage, sex, rightsidedness and mucinous differentiation. Only microsatellite stable (MSS) tumours were included because MSI-tumours already have better prognosis in low-stage colorectal cancers [28,29]. Furthermore, only elective surgery, tumours without perforation and/or spill were selected. FFPE whole tissue sections were collected from all 46 tumours. In brief, these FFPE tissues were dissected and prepared for liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis. Proteins were identified by MS/MS spectra using MaxQuant and subsequently quantified by spectral counting. More details are described in the supplementary methods, SM2 (appendices, chapter 9). The normalised counts for CDX2 per patient were used for statistical purposes. Dichotomisation using ROC analysis resulted in 'CDX2-high' annotation when levels exceeded 2.19 normalised counts.

Statistical analysis

Chi-square, Fisher's exact and Mann-Whitney U statistical tests were used to analyse differences between study groups, where appropriate. Hazard ratio (HR) with 95% confidence interval (CI) were calculated using Cox regression analysis. DSS and DFS were visualised by Kaplan-Meier curves, and p-values were obtained from log-rank tests. Multivariable analysis was performed using stepwise backwards Cox regression, and included expression of CDX2 and clinicopathological parameters. Correlation between expression levels of both techniques was analysed using ANOVA and linear trend analysis. The discriminatory power of CDX2 expression using either of the two techniques was determined by calculating the AUC, that is, the area under the ROC curve. The difference in discriminatory power was tested using the deLong method for a paired design [30,31]. More details are described in supplementary methods, SM3 (appendices, chapter 9).

RESULTS

Low expression of CDX2 based on immunohistochemical evaluation is associated with poor DSS

Immunohistochemical staining of CDX2 protein expression could be evaluated in 346 patients. CDX2 protein expression levels were categorically annotated as strong, moderate, weak and negative **(Figure 1A-D)** and associated with DSS **(Figure 1E)**. These data illustrate that a gradual decline in CDX2 protein levels tends to be associated with increasingly poor survival rates. Dichotomised scores of CDX2 expression were used for further analyses. One hundred ninety-two tumours were CDX2-low (55.5%) and 154 were CDX2-high (44.5%). There were no significant differences in baseline clinical characteristics between the CDX2-low and CDX2-high expression group, apart from survival (**Table 1**). MSI tumours tended to be more prevalent in the CDX2-low compared with the CDX2-high expression group (19.8% versus 11.0%; p = 0.053). Low expression of CDX2 was associated with poor DSS in 346 patients with stage II and III colon cancer (HR = 1.97 [95% CI: 1.26-3.06]; p=0.002) **(Figure 2A)**.

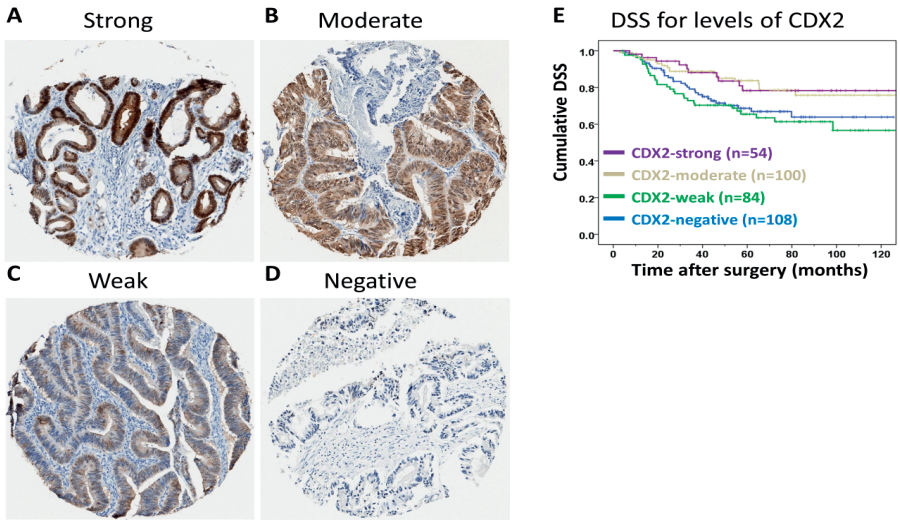

Figure 1: CDX2 protein expression in stage II and III colon cancer and its association with DSS. Representative examples indicative for strong (A), moderate (B), weak (C) and negative (D) immunohistochemical staining intensities of CDX2 expression. Survival of CDX2 protein expression levels was visualized with Kaplan Meier curves for DSS (E).

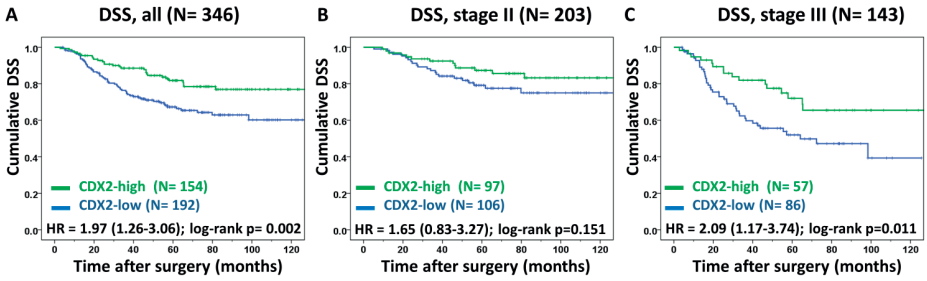

Figure 2: Association of CDX2 expression based on immunohistochemical detection with DSS of colon cancer patients. Stratification for CDX2-high and CDX2-low expression in all stage II and III colon cancer patients (A); in stage II colon cancer patients (B); and in stage III colon cancer patients (C). Cox regression HR (95% CI) and log-rank P-values are reported.

Table 1: Baseline characteristics and clinicopathological data stratified for low and high MVD for both stage II and III colon cancer. P-values were calculated by chi-square test or independent t-testing for continuous data. Significant p-values are printed in bold.

	Stage II: n= 53 (%)			**Stage III: n= 54 (%)**		
Clinicopathological characteristics	**MVD-low n= 26 (%)**	**MVD-high n= 27 (%)**	**p-value**	**MVD-low n= 15 (%)**	**MVD-high n= 39 (%)**	**p-value**
Sex						
Male	14 (53.8)	14 (51.8)		10 (66.7)	25 (64.1)	
Female	12 (46.2)	13 (48.2)	0.88	5 (33.3)	14 (35.9)	0.86
Age, mean (s.d.) (years)	73.7 (11.3)	70.9 (13.3)	0.41	63.6 (9.8)	66.6 (10.1)	0.32
Right sided tumour	9 (34.6)	8 (29.6)	0.70	7 (46.7)	18 (46.2)	0.97
Diameter, mean (s.d.) (mm)	39.2 (20.7)	40.80 (19.3)	0.61	36.4 (10.5)	33.9 (13.4)	0.44
Histological grade						
Good	2 (7.7)	3 (11.1)		1 (6.7)	1 (2.6)	
Average	23 (88.5)	23 (85.2)		12 (80.0)	35 (89.7)	
Poor	1 (3.8)	1 (3.7)	0.91	2 (13.3)	3 (7.7)	0.61
Tumour stage						
T1	-	-		-	1 (2.6)	
T2	-	-		1 (6.7)	6 (15.4)	
T3	23 (88.5)	26 (96.3)		10 (66.7)	30 (76.9)	
T4	3 (11.5)	1 (3.7)	0.28	4 (26.7)	2 (5.1)	0.13
Nodal stage						
N1	-	-		12 (80.0)	22 (56.4)	
N2	-	-	-	3 (20.0)	17 (43.6)	0.11
Mucinous differentiation.	8 (30.8)	4 (14.8)	0.17	1 (6.7)	4 (10.3)	0.68
Ulceration	18 (69.2)	23 (85.2)	0.17	10 (66.7)	35 (89.7)	**0.042**
Angioinvasion	2 (7.7)	4 (14.8)	0.41	7 (46.7)	14 (35.9)	0.47
Perforation						
No	24 (92.3)	24 (88.9)		14 (93.3)	37 (94.9)	
Before surgery	1 (3.8)	1 (3.7)		1 (6.7)	1 (2.6)	
During surgery	-	1 (3.7)		-	-	
After surgery	1 (3.8)	1 (3.7)	0.81	-	1 (2.6)	0.64
Tumour spill	1 (3.8)	2 (7.4)	0.58	1 (6.7)	-	0.10
Adjuvant chemo	0	0	-	15 (100.0)	39 (100.0)	-
Recurrence	6 (23.1)	12 (44.4)	0.10	11 (73.3)	14 (35.9)	**0.013**
CRC mortality	6 (23.1)	9 (33.3)	0.41	8 (53.3)	12 (30.8)	0.12
Overall mortality	14 (53.8)	16 (59.3)	0.69	9 (60.0)	17 (43.6)	0.28
Follow up, mean (s.d.) months	70.5 (32.6)	58.1 (35.9)	0.19	52.3 (33.7)	57.7 (27.4)	0.54

Separate analyses for stage II (N = 203) and III (N = 143) revealed similar survival curves, although statistical significance was only reached for patients with stage III colon cancer (HR = 2.09 [95% CI: 1.17-3.74]; p=0.011) **(Figure 2B-C).** Further stratification of patients with stage II and stage III colon cancer into subgroups who did or did not receive ACT yielded similar results **(Supplementary Figure 1A-D,** appendices, chapter 9**).** Among the cancers for which MSI status was known, similar HRs were observed for MSI and MSS cancers, although the association with DSS was not significant for the subgroup of 55 MSI cancers (HR = 1.81 [95% CI: 0.50-6.57]; p=0.36), whereas it was significant for the subgroup of 241 MSS cancers (HR = 2.09 [95% CI: 1.26-3.46]; p=0.004) **(Supplementary Figure 2,** appendices, chapter 9**).** Moreover, no association was found between CDX2 expression levels and DFS **(Supplementary Figure 3A-C,** appendices, chapter 9**).** Multivariable Cox regression showed that low expression of CDX2 was independently associated with poor DSS, (HR = 1.85 [95% CI: 1.15-2.97]; p=0.011), in addition to the prognostic parameters T stage, angioinvasion, tumour spill and stage III **(Table 2).**

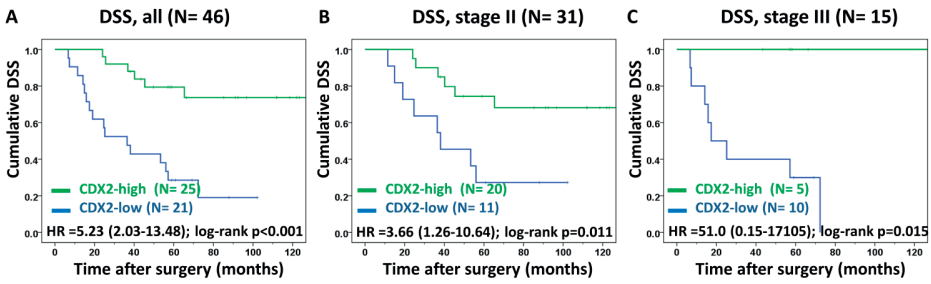

Figure 3: Association of CDX2 expression based on mass spectrometry with DSS of colon cancer patients. Stratification for CDX2-high and CDX2-low expression in all stage II and III colon cancer patients (A); in stage II colon cancer patients (B); and in stage III colon cancer patients (C). Cox regression HR (95% CI) and log-rank P-values are reported. Note that for stage III no reliable HR could be obtained, since there were no events in the CDX2-high group.

Table 2: Multivariate backward Cox-regression analysis for 5-year disease free survival of high microvessel density and clinicopathological parameters that were retained in the model, in stage II and III colon cancer patients. HR: Hazard ratio; 95%-CI: 95% confidence interval.

Stage	Parameters	HR	95%-CI	P-value
II	Right-sided tumour	0.18	0.04-0.83	0.027
	Ulceration	6.80	0.89-52.23	0.065
	High microvessel density	**4.50**	**1.38-14.64**	**0.013**
III	Ulceration	0.32	0.10-1.06	0.062
	Angioinvasion	4.38	1.71-11.23	0.002
	High microvessel density	**0.34**	**0.13-0.90**	**0.031**

Low expression of CDX2 based on LC-MS/MS is associated with poor DSS

This mass spectrometry analysis was performed in a nested case-control cohort of 46 patients consisting of stage II and III colon cancer patients who were not treated with ACT. Normalised counts for CDX2 ranged from 0 to 6.53, with a non-normal distribution (Shapiro- Wilk test, p = 0.029) and a median of 2.48. Dichotomised scores of CDX2 expression were used forfurther analyses. Twenty-five tumours were CDX2-high (54.3%) and 21 were CDX2-low (45.7%). There were no significant differences in baseline clinical characteristics between the CDX2-low and CDX2-high groups, apart from survival **(Table 3).** Low expression of CDX2 was associated with poor DSS (HR = 5.23 [95% CI: 2.03-13.48]; p < 0.001) **(Figure 3A).** Similar results were observed for the 31 patients with stage II (HR = 3.66 [95% CI: 1.26-10.64]; p=0.011) and 15 patients with stage III colon cancer (HR = 51.03 [95% CI: 0.15-17,105]; p=0.015) **(Figure 3B and C).** Please note that the exact HR in the subset of patients with CDX2-high stage III colon cancer is unreliable due to the fact that all patients were event-free up to at least 43 months, and the number of patients is low. Similar results were obtained for association between CDX2 expression levels and DFS **(Supplementary Figure 4A-C,** (appendices, chapter 9)**.** Multivariable Cox regression analysis showed that low levels of CDX2 were independently associated with poor DSS (HR = 13.7 (95% CI 4.35-43.27); p<0.001), in addition to histological grade, ulceration and angioinvasion **(Table 4).**

Table 3: Clinicopathological characteristics of 46 patients with colon cancer, stratified for low and high protein mass spectrometry counts of CDX2. Significant differences between the CDX2-low and CDX2-high group are presented in bold, based on Fisher's exact or Mann-Whitney U test.

Clinicopathological characteristics	Total: n= 46 (%)	Low-CDX2 n= 21 (%)	High-CDX2 n=25 (%)	p-value
Sex				
Male	23 (50.0	10 (47.6)	13 (52.0)	
Female	23 (50.0)	11 (52.4)	12 (48.0)	1.0
Age, median (range) (yr)	76.2 (60.7-91.5)	77.7 (60.7-87.4)	71.0 (61.8-91.5)	0.18
Right sided tumor	21 (45.7)	11 (52.4)	10 (40.0)	0.55
Histological grade				
Well	3 (6.5)	1 (4.8)	2 (8.0)	
Moderate	39 (84.8)	17 (81.0)	22 (88.0)	
Poor	4 (8.7)	3 (14.3)	1 (4.0)	0.47
Tumor stage				
T1	-	-	-	
T2	2 (4.3)	0 (0.0)	2 (8.0)	
T3	40 (87.0)	19 (90.5)	21 (84.0)	
T4	4 (8.7)	2 (9.5)	2 (8.0)	0.66
Nodal stage				
N0	31 (67.4)	11 (52.4)	20 (80.0)	
N1	8 (17.4)	6 (28.6)	2 (8.0)	
N2	7 (15.2)	4 (19.0)	3 (12.0)	0.09
Stage				
II	31 (67.4)	11 (52.4)	20 (80.0)	
III	15 (32.6)	10 (47.6)	5 (20.0)	0.063
Mucinous differentiation.	13 (28.3)	7 (33.3)	6 (24.0)	0.53
Isolated tumor deposits	6 (13.0)	4 (19.0)	2 (8.0)	0.39
Ulceration	36 (78.3)	16 (76.2)	20 (80.0)	1.0
Angioinvasion	9 (19.6)	5 (23.8)	4 (16.0)	0.71
Tumor spill	1 (2.2)	1 (4.8)	0 (0.0)	0.46
Adjuvant chemotherapy	1 (2.2)	1 (4.8)	0 (0.0)	0.27
Recurrence	23 (50.0)	16 (76.2)	7 (28.0)	**0.001**
CRC mortality	22 (47.8)	16 (76.2)	6 (24.0)	**<0.001**
Overall mortality	27 (58.7)	16 (76.2)	11 (44.0)	**0.027**

Table 4: Multivariable cox regression with backward variable selection for low counts of CDX2 in mass spectrometry analysis for disease-specific survival (DSS).

Parameters	HR	95%-CI	P-value
Loss of CDX2	**13.7**	**4.35 – 43.27**	**<0.001**
Histological grade	8.38	2,21 – 31.77	0.002
Ulceration	5.29	1.41 – 19.91	0.014
Angioinvasion	3.88	1.30 – 11.64	0.015

Improved prognostic value of CDX2 expression when determined by tandem mass spectrometry proteomics

To performa head-to-head comparison of the prognostic value of CDX2 when determined by immunohistochemistry versus by LC-MS/MS tandem mass spectrometry, analyses were focused on the 41 cases that were evaluated by both methodologies. Increasing expression levels of CDX2 as determined by immunohistochemistry are associated with significantly higher levels of CDX2 as determined by mass spectrometry (ANOVA and linear trend analysis; p=0.047). Nevertheless, for this subset of patients, immunohistochemical evaluation of CDX2 protein expression showed no significant association with DSS (HR = 1.60 [95% CI: 0.61-4.22]; p=0.339) **(Figure 4A)**, whereas LC-MS/MS-based proteomics evaluation of CDX2protein expression of the same subset of 41 patients did show a highly significant association with DSS (HR = 7.56 [95% CI: 2.49-22.95]; p<0.001 **(Figure 4B)**.

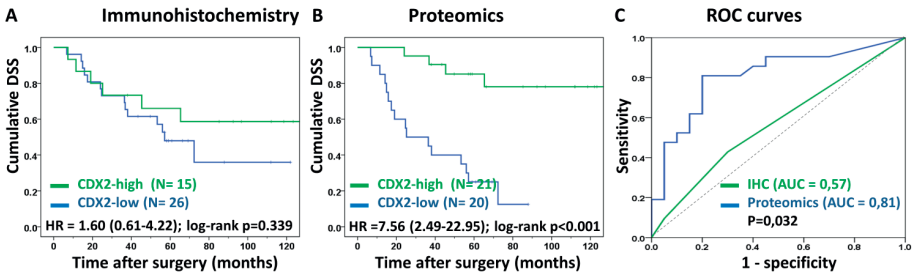

Figure 4: Association of DSS with CDX2 expression based on immunohistochemical detection (A) and mass spectrometry (B) in the same subset of 41 patients. Cox regression HR (95% CI) and log-rank P-values are reported. ROC curves (C) of both techniques for the subs et of 41 patients are presented with corresponding areas under the curve (AUC) and comparison according to deLong method for a paired design (30).

Similar observations were made when patients were analysed separately for stage II and III **(Supplementary Figure 5A-D,** appendices, chapter 9**).** Then, ROC curves were generated to compare the CDX2 assay performance, which indicated that the discriminatory power of mass spectrometry (AUC = 0.81 [95% CI: 0,67-0.95]) was significantly higher than routine immunohistochemistry (AUC= 0.57 [95% CI: 0.39-0.75]; p=0.032; **Figure 4C**).

DISCUSSION

In the present study, the prognostic value of CDX2 was validated in a well-described cohort of patients with stage II and III colon cancer. When detecting CDX2 protein expression by immunohistochemistry using an assay that is currently operational in many pathology laboratories, low expression of CDX2 was associated with poor prognosis. The independent prognostic value of CDX2 remained significant in a multivariate analysis. Furthermore, the HRs obtained from immunohistochemical evaluation of CDX2 in this study (approximately 2.0) are in accordance to those reported previously, reaching approximately 2.4 at best [20,22,23]. Only for stage IV disease, higher HRs have been reported [32]. However, with HRs less than 3, the clinical utility of CDX2 is limited and may be considered insufficient for clinical implementation as a prognostic biomarker. The fact that these data confirmed earlier observations lends further confidence to CDX2 as a prognostic biomarker in stage II and III colon cancer [20-23].

Determination of the actual prognostic value of CDX2 in the present study might be limited by putative confounding factors. For instance, MSI tumours tended to be more prevalent in the CDX2-low compared with the CDX2-high expression group **(Table 1).** It is of interest to note that localised MSI colon cancers have a better prognosis than localised MSS colon cancers (see also **Supplementary Figure 2,** appendices, chapter 9). This means that the putative confounding effect of MSI status, if any, would result in an underestimation of the prognostic value of CDX2. Similarly, patients with a poor disease outcome are more prevalent in the CDX2-low compared with the CDX2- high expression group. Consequently, relatively more patients in the CDX2-low expression group received additional therapy. Assuming that the subsequent therapies prolonged patient survival, the actual prognostic value of CDX2 might be underestimated.

One of the limitations of immunohistochemistry is that there is no linear correlation between protein expression levels and staining intensity, causing this methodology to be semiquantitative in nature and forcing pathologists to annotate expression levels in a categorical manner. With such an approach, the true discriminatory power of a putative prognostic biomarker may be lost. We therefore examined the prognostic value of CDX2 using a more quantitative detection method that would yield continuous data and determined CDX2 protein expression by LC-MS/MS tandem mass spectrometry proteomics. Interestingly, in a head-to-head comparison, the HR of mass spectrometryebased CDX2 scores was much higher (HR = 7.56) than the immunohistochemistry-based HR (HR = 1.60). Visualisation of assay performance by plotting ROC curves revealed that the AUC of mass spectrometry was significantly larger than that of routine immunohistochemistry, implying that replacement of routine immunohistochemistry by a more quantitative assay can improve the discriminatory power of a (CDX2 prognostic) biomarker assay, thereby increasing its clinical utility.

Immunohistochemistry is a key technology in diagnostic pathology, not in the least because it can be evaluated by microscopy. Yet, executing immunohistochemistry in the lab and interpreting and scoring the staining patterns is not without problems. While immunohistochemistry has become a fixed value in the armamentarium for making differential diagnoses, this is much less the case so for prognosis and response prediction. Efforts in standardisation and quality assurance are not always able to overcome this problem. For one, the amount of staining ultimately observed under the microscope is not linearly proportional to the amount of protein present in the tumour cells, i.e. immunohistochemistry is not stoichiometric. This is due to the fact that the protein expression signal is substantially enhanced during the staining procedure, the effect of which is dependent of many pre-analytical variables at almost all levels of the lab process, which vary substantially between labs. Technically, between hospitals and studies, there might be variation in tumour tissue fixation protocols, clones and batches of antibodies used, staining reagents and staining protocols. As a consequence, scoring systems of immunohistochemistry are rather crude with at best only a few discrete classes, which may lead to different distribution of expression-scores between laboratories and studies. An advantage of immunohistochemistry is that spatial and (sub)cellular distribution of a marker can be taken into account, by scoring tissue components such as epithelial cells or

stroma or cellular components such as nuclei or cytoplasm. For CDX2, expression is confined to the nuclei of epithelial cells in both normal colon and cancer, allowing evaluation of its expression levels using methodologies that are not highly dependent on tissue morphology or subcellular distribution.

The present study resulted in better performance of CDX2 as a prognostic biomarker when measured by LCMS/MS tandem mass spectrometry compared with immunohistochemistry, implying that quantitative assays for detection of CDX2 may be better suited to determine its prognostic value than immunohistochemistry. Therefore, quantitative assays such as Enzyme-Linked Immuno Sorbent Assay (ELISA) or targeted mass spectrometry assays such as selective reaction monitoring (SRM) or parallel reaction monitoring (PRM) should be considered as an alternative for immunohistochemistry to facilitate successful clinical implementation. Data-independent acquisition mass spectrometry is another example of an emerging highthroughput technology with the potential to be robustly applied with high reproducibility of quantitative proteomics data [33]. After such an appropriate quantitative assay has been developed, the biomarker has to be validated prospectively. Subsequently, multicentre clinical trials with randomisation for adjuvant treatment have to be organised while taking cost-effectiveness into account. Only once the clinical utility has been proven and 'more health at less cost' can be demonstrated, biomarker implementation in standard of care is likely to be achieved.

CONCLUSION

In conclusion, this study confirmed once more that CDX2 protein expression is a prognostic biomarker for patients with stage II and stage III colon cancer. Importantly, we here demonstrate that the discriminatory power of CDX2 as a prognostic biomarker is much higher when measured by a quantitative assay compared to its detection by routine immunohistochemistry. Therefore, when considering to evaluate the clinical utility of CDX2 expression in stage II or III colon cancer in a biomarker-driven clinical trial, quantitative assays should be considered as an alternative for immunohistochemistry to increase chances towards successful clinical implementation.

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CHAPTER 4

Loss of KCNQ1 expression in stage II and stage III colon cancer is a strong prognostic factor for disease recurrence.

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ABSTRACT

Background

Colorectal cancer (CRC) is the third most common cancer worldwide. Accurately identifying stage II CRC patients at risk for recurrence is an unmet clinical need. KCNQ1 was previously
identified as a tumour suppressor gene and loss of expression was associated with poor survival in patients with CRC liver metastases. In this study the prognostic value of KCNQ1 in stage II and stage III colon cancer patients was examined.

Methods

KCNQ1 mRNA expression was assessed in 90 stage II colon cancer patients (AMC-AJCCII-90) using microarray gene expression data. Subsequently, KCNQ1 protein expression was evaluated in an independent cohort of 386 stage II and stage III colon cancer patients by immunohistochemistry of tissue microarrays.

Results

Low KCNQ1 mRNA expression in stage II microsatellite stable (MSS) colon cancers was associated with poor disease-free survival (DFS) (P=0.025). Loss of KCNQ1 protein expression from epithelial cells was strongly associated with poor DFS in stage II MSS (P<0.0001), stage III MSS (P=0.0001) and stage III microsatellite instable colon cancers (P=0.041). KCNQ1 seemed an independent prognostic value in addition to other high-risk parameters like angio-invasion, nodal stage and microsatellite instability-status.

Conclusions

We conclude that KCNQ1 is a promising biomarker for prediction of disease recurrence and may aid stratification of patients with stage II MSS colon cancer for adjuvant chemotherapy.

INTRODUCTION

With over 1.3 million new patients a year and an estimated 694,000 deaths in 2012, colorectal cancer (CRC) is an increasing health-care issue, especially in more developed countries [1]. Current guidelines for treatment of primary non-metastatic colon cancer prescribe surgery and adjuvant systemic therapy, the latter based on pathological staging and clinical features. There is consensus that adjuvant chemotherapy generally improves survival in stage III CRC patients without significant comorbidities [2-4]. For stage II CRC patients this is less obvious and only patients with high risk features may benefit from adjuvant treatment [5-7]. However, the definition of these high risk features remains subject of debate. The high risk clinicopathological features as defined by ASCO-guidelines are perforation or obstruction at presentation, T4, no or poorly differentiated, vascular invasion or inadequate amount of harvested lymph nodes [5]. With 5-year survival rates ranging from 72-83% for stage II and 44-83% for stage III CRC patients, approximately 25% of stage II CRC patients who do not receive chemotherapy will have disease recurrence. Furthermore, a substantial proportion of stage III patients suffers from the side effects of chemotherapy although they might not develop recurrences even if they wouldn't receive adjuvant therapy [8]. Therefore, there is a clear clinical need for better prognostic markers to more accurately identify stage II patients at high risk and stage III patients at low risk of disease recurrence.

Clinical disease course is driven by tumour biology and biomarkers therefore have the potential to complement and improve prognostic value of current clinicopathological features. One example of such a biomarker is microsatellite instability (MSI), which is associated with a more favourable prognosis in stage I and II disease compared to microsatellite stable (MSS) tumours [9-12]. Within the group of MSS patients, which comprises 80-85 % of all CRCs, there are still major differences in survival, so further stratification is needed for adequate selection of patients who may benefit from adjuvant chemotherapy. Recently, several studies succeeded to classify CRC patients into subgroups with relatively good or poor prognosis, in particular based on RNA signatures [13-19]. However, clinical implementation of such gene signatures faces practical hurdles because collection and processing of fresh-frozen tumour samples for RNA isolation is not an established routine workflow in most hospitals, and

is logistically demanding. One solution to this problem is to identify prognostic biomarkers that can be implemented in existing routine clinical workflows, such as protein biomarkers that can be evaluated by immunohistochemical staining of formalin-fixed paraffin-embedded (FFPE) tissue material.

We previously identified *KCNQ1* as a tumour suppressor gene in mouse and human gastrointestinal cancers [20]. The *KCNQ1* gene is located on chromosome 11p15.5 and encodes for the α-subunit of voltage-gated potassium channels. KCNQ1 has mainly been studied for its function in the basolateral cell membrane and its predominant presence in cardiac tissue [21], but recently its role in tumourigenesis has gained interest [20,22,23]. It was postulated that *Kcnq1* mutations play a role in the development of metaplasia, dysplasia and pre-malignant adenomatous hyperplasia and gastric cancer [24]. In colorectal cancer, *Kcnq1* ranked among the highest common insertion site genes in several sleeping beauty DNA-transposon based forward genetic screens, indicating its putative role as a cancer driver gene [22,23,25]. This was further confirmed by studies of *Kcnq1* knockout mice, which exhibited enhanced intestinal tumour multiplicity and tumour progression. Moreover, loss of *KCNQ1* expression in human CRC liver metastases was associated with poor prognosis [20]. Interestingly, β-catenin has been described to regulate KCNQ1 protein expression in cardiac and gastric tissue [26,27]. Indeed, activation of the Wnt signalling pathway *in vivo* by conditional deletion of *APC*, in which β-catenin plays a crucial role, resulted in increased expression of well-known Wnt-target genes such as *CD44* as well as increased expression of *KCNQ1* [28].

So far, the role of *KCNQ1* in colon cancer patients who lack distant metastases has not been established. Therefore, the present study aimed to determine the prognostic value of *KCNQ1* in stage II and III colon cancer patients. We here report that loss of KCNQ1 protein expression is a strong prognostic factor for recurrence and survival in stage II and III colon cancer patients.

MATERIALS AND METHODS

Patient study populations

The present study made use of two previously published patient series to examine associations of *KCNQ1* to disease free survival (DFS). The AMC-AJCCII-90 series consisted of 90 stage II colon cancer patients from the Academic Medical Center in Amsterdam, the Netherlands [14], and was used to investigate association of *KCNQ1* mRNA expression to DFS. The second series consisted of 226 stage II and 160 stage III colon cancer patients from the Kennemer Gasthuis in Haarlem, the Netherlands [29], and was used to investigate the relation between KCNQ1 protein expression and DFS. All CRC cases were sporadic except for one patient with Lynch syndrome (HNPCC) and one patient with familial adenomatous polyposis (FAP) in the AMC-cohort. Both study populations have previously been characterised for MSI status by a 5-marker based PCR analysis system, as described previously [14,30]. Collection, storage and use of clinicopathological data and tissue specimens were performed in compliance with the 'Code for Proper Secondary Use of Human Tissue in The Netherlands' [31].

KCNQ1 mRNA expression analysis

The AMC-AJCCII-90 cohort was previously characterised for genome-wide gene expression by microarray analysis (GSE33113)[14]. For the present study, *KCNQ1* mRNA expression in tumour tissue was dichotomised in low and high expression using a cut-off that was determined based on optimal separation (R2 platform, http://hgserver1.amc.nl/). Hazard ratios (HR), 95% confidence intervals (95% CI) and p-values were calculated using Cox regression analysis. DFS survival curves were visualised by Kaplan-Meier curves and compared using the log-rank test.

KCNQ1, β-catenin, and CD44 protein expression analysis

The Kennemer Gasthuis series of 386 stage II and III colon cancers was previously used to generate tissue microarrays (TMAs)[29]. In brief, three core biopsies (diameter 0.6 mm) were obtained from the centre and three from the periphery of each FFPE tumour tissue donor block and inserted into recipient TMA paraffin blocks using the 3DHISTECH TMA Master (v1.14, 3DHISTECH Ltd, Budapest, Hungary). Protein expression was evaluated by immunohistochemical staining of TMAs. 4 µm sections were mounted on glass slides, deparaffinised by xylene

and rehydrated with a decreasing alcohol series. Staining for KCNQ1 was performed following antigen retrieval by microwave heating in citric acid (10 mM, pH 6.0) and endogenous peroxidase neutralization in 0.3% hydrogen peroxide in methanol for 25 min, as described previously [20]. The primary rabbit polyclonal antibody directed against human KCNQ1 (sc-20816; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was incubated overnight at a 1:200 dilution at 4 °C, followed by incubation with anti-rabbit secondary antibodies for 30 min at room temperature (Envision Plus; Dako, Heverlee, Belgium). Secondary antibodies were visualised by liquid diaminobenzidine substrate chromogen system. Slides were counterstained with Mayer's hematoxylin. FFPE colon tissue was used as positive control whereas incubation without primary antibody was used as negative control. Staining for β-catenin (CTNNB1) was performed upon antigen retrieval by microwave heating in Tris buffer (pH 9.0) and endogenous peroxidase neutralization in 0.3% hydrogen peroxide in methanol for 25 min, followed by incubation with a mouse monoclonal antibody directed against human β-catenin (NCL-B-CAT, clone 17c2, Menarini, Florence, Italy) in a 1:100 dilution for one hour at room temperature. Staining for CD44 was performed upon antigen retrieval by microwave heating in citric acid (pH 6.0) and endogenous peroxidase neutralization in 0.3% hydrogen peroxide in methanol for 25 min, followed by incubation with a monospecific polyclonal rabbit antibody was used (HPA005785, Atlas antibodies, Stockholm, Sweden) in a 1:150 dilution for one hour at room temperature. β-catenin and CD44 antibody binding was detected using Brightvision+ (Lot.no 80430J, Immunologic, Duiven, The Netherlands).

Immunohistochemical stainings were digitally captured using the Mirax slide scanner system equipped with a 20x objective with a numerical aperture of 0.75 (Carl Zeiss BV, Sliedrecht, The Netherlands) and a Sony DFW-X710 Fire Wire 1/3 in-type progressive SCAN IT CCD (pixel size 4.65 × 4.65 µm2). Actual scan resolution at 20x was 0.23 µm, as described previously [29]. TMA core biopsies were scored for neoplastic epithelial cell membrane staining intensity of KCNQ1 and CD44, and nuclear staining intensity of β-catenin (categories negative, weak, moderate, strong), using dedicated TMA scoring software (v.1.14.25.1; 3DHISTECH Ltd). Damaged and missing cores, defined as less than 1/3rd of core remaining, were not scored. Highest scores on intensity for each patient were converted to a clinical SPSS database. Since no differences were observed between tumour central and peripheral locations for KCNQ1, CD44

and β-catenin, these cores were combined for further analysis. In this way, 377 patients could be assessed for KCNQ1 staining, 372 patients for CD44 staining, and 378 patients for β-catenin staining.

Protein expression scores for KCNQ1, β-catenin, and CD44 were dichotomised for analysis of patient subgroups. First, the data was randomly split into five subsets. Next, the optimal cut-off for dichotomizing scores into a high- or low-expression group was based on 4/5th of the dataset using Receiver Operating Characteristic (ROC) curve analysis for survival data with 5-year DFS as the outcome of interest. This procedure was repeated five times, with 1/5th of the dataset varying. The final cut-off was the cut-off that was most often selected. In this way, the optimal cut-off for KCNQ1 was set to 'low expression' for negative and weak intensity scores and 'high expression' for moderate and strong intensity scores. The optimal cut-off for β-catenin was set to 'low expression' for negative and 'high expression' for weak, moderate and strong intensity scores. For CD44 the optimal cut-off was set to 'low expression' for negative, weak and moderate intensity scores and 'high expression' for strong intensity scores.

Statistical evaluation

Differences in baseline characteristics between the groups with high and low expression of KCNQ1 were analysed using the independent-t-test in case of continuous variables, taking Levene's test for equality of variances into account. The Pearson's chi-square test (or Fisher's exact test when appropriate) was used to examine associations between dichotomous or categorical variables. HR, 95% CI, and p-values were calculated using Cox regression analysis. High expression was used as the reference category for each of the three biomarkers. DFS was visualised by Kaplan Meier curves and compared using the log-rank test. Multivariate analysis was performed using stepwise backwards Cox regression, with DFS as dependent variable and an exclusion criterion of p>0.1. Based on the 'rule of ten' only a limited number of variables were allowed comprising KCNQ1 and 11 other parameters including previously identified prognostic variables. These parameters were tumour location (right sided), T- and N-stage, stage, isolated tumour deposits, angioinvasion, grade of differentiation, ulceration, perforation, tumour spill, and MSI status [5,10,32,33]. Associations between expression of KCNQ1 and β-catenin or CD44 expression were analysed with the chi-square test. All statistical tests were two-sided.

p-values < 0.05 were considered significant. Analyses were performed using SPSS (IBM SPSS Statistics, SPSS Inc., v.20.0 for windows, Chicago, Illinois, USA).

RESULTS

Low KCNQ1 mRNA expression is associated with poor DFS in stage II MSS colon cancer

To investigate prognostic value of *KCNQ1* mRNA expression in nonmetastatic CRC patients, we made use of existing gene expression data from the AMC-AJCCII-90 study population consisting of 90 stage II colon cancer patients (14). Overall, no significant association between *KCNQ1* mRNA expression levels and DFS was observed (**Figure 1A)**.

Because MSI status is a known confounding factor with prognostic value in stage II CRC patients [10,11], the association between *KCNQ1* mRNA expression and DFS was analysed in MSS (n=65) and MSI tumours (n=25) separately. Low expression of *KCNQ1* was associated with poor survival in patients with stage II MSS tumours (HR = 3.35 [95% CI: 1.16-9.66]; p=0.025) (**Figure 1B**) but not in patients with MSI tumours (HR = 0.24 [95% CI: 0.03-2.15]; p=0.20) (**Figure 1C**). These mRNA data indicate that *KCNQ1* has prognostic potential in non-metastatic CRC, and prompted us to further validate these findings. Because determination of protein expression by immunohistochemistry is part of existing clinical workflows, loss of KCNQ1 protein expression in neoplastic epithelial cells was analysed subsequently.

Loss of KCNQ1 expression is associated with poor DFS in stage II/III MSS and stage III MSI colon cancer patients

Prognostic value of KCNQ1 protein expression by neoplastic cells was examined by immunohistochemical evaluation of TMAs containing tumour tissue from 226 stage II and 160 stage III colon cancer patients **(Figures 2A-D)**. Association between KCNQ1 staining intensity and DFS is clearly demonstrated in **Figure 2E**. This figure shows that decreasing amounts of epithelial KCNQ1 expression were associated with increasingly worse prognosis.

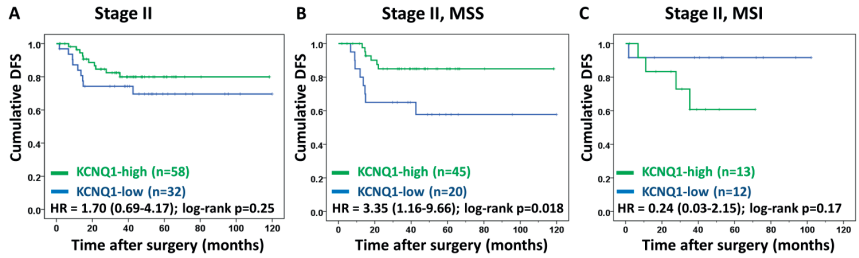

Figure 1: Association of KCNQ1 mRNA expression with DFS of (A) stage II colon cancer patients (AMC-AJCCII-90 study population); (B) the subset of MSS patients; and (C) the subset of MSI patients. Cox regression HR (95% CI) and log-rank P-values are reported.

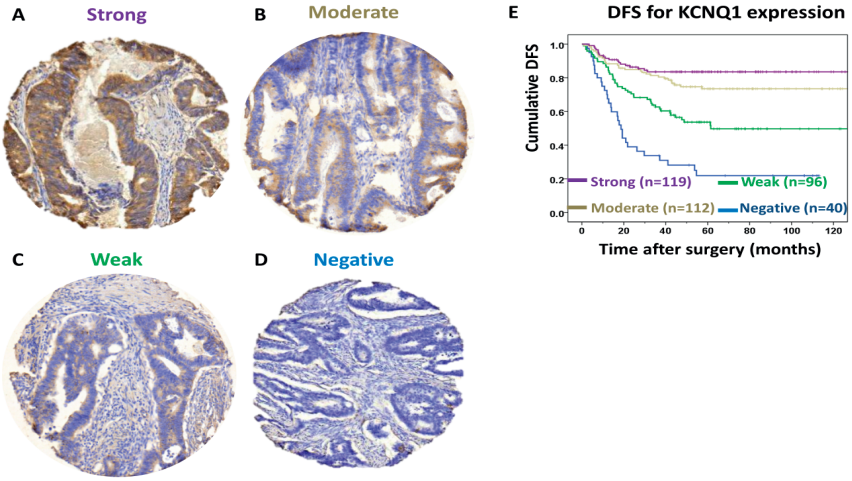

Figure 2: KCNQ1 protein expression and its association with DFS of stages II and III colon cancer patients. (A–D) Representative examples ((A) strong, (B) moderate, (C) weak and (D) negative) of immunohistochemical staining intensities of KCNQ1 expression. (E) Association of KCNQ1 protein expression levels with DFS.

To analyse the effects of KCNQ1 in subgroups of colon cancer patients, protein expression data were dichotomized into 'low' and 'high' KCNQ1 expression. Low KCNQ1 expression was associated with poor DFS in stage II colon cancer patients (HR = 3.61 [95% CI: 2.07-6.33]; p< 0.0001) **(Figure 3A)** and in stage III colon cancer patients (HR = 3.11 [95% CI: 1.93-5.01]; p=0.0001) **(Figure 3D)**. Because MSI status is a known confounding factor [10,11], the prognostic value

of KCNQ1 expression was also analysed in stage II and III MSS and MSI patients separately. MSI status had previously been determined for 325 patients of this cohort, revealing 63 MSI (19%) and 262 MSS (81%) colon tumours [30]. In the group of MSS tumours, low expression of KCNQ1 was associated with poor DFS in both stage II colon cancer patients (HR = 3.82 [95% CI: 2.04-7.14]; p<0.0001) **(Figure 3B)** and stage III colon cancer patients (HR = 2.93 [95% CI: 1.70-5.0]2; p<0.0001) **(Figure 3E).** In the group of MSI tumours, low expression of KCNQ1 was not significantly associated with DFS in stage II colon cancer patients (HR = 3.37 [95% CI: 0.38-30.17]; p=0.278) **(Figure 3C)**, but was associated with poor DFS in stage III colon cancer patients (HR = 5.06 [95% CI: 1.07-23.89]; p=0.041) **(Figure 3F)** colon cancer patients.

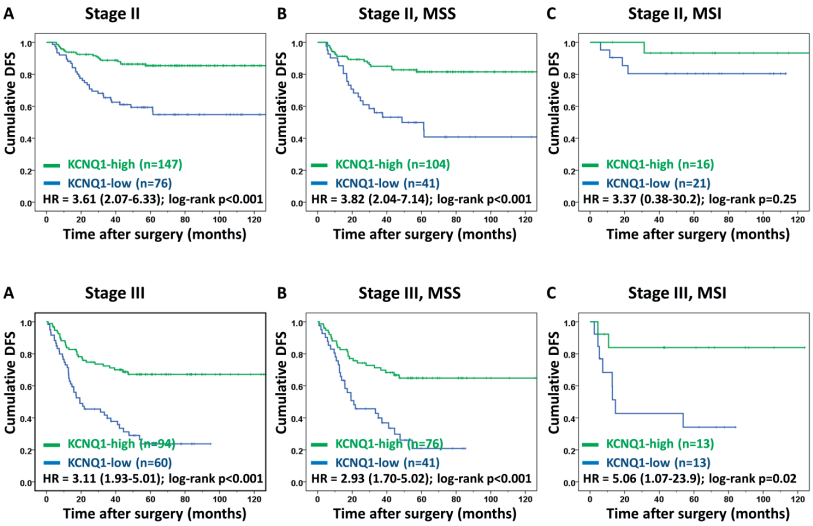

Figure 3: DFS for stage II (A–C) and III (D–F) colon cancer patients, stratified for high- and low-expression of KCNQ1 in all patients (A and D), the subset of MSS patients (B and E) and the subset of MSI patients (C and F). Cox regression HR (95% CI) and log-rank P-values are reported.

Loss of KCNQ1 expression is associated with poor DFS in stage III MSS colon cancer patients stratified for adjuvant chemotherapy

A large subset of patients had been treated with 5FU-based adjuvant chemotherapy, which is likely to affect survival. Therefore, the association between

KCNQ1 expression and DFS was analysed separately in subgroups of stage II and III patients who did or did not receive adjuvant chemotherapy. Only MSS patients were analysed, because the number of MSI patients was too small for further analysis of patient subgroups. In patients who did not receive chemotherapy, low expression of KCNQ1 was associated with poor DFS in stage II patients (HR = 4.06 [95% CI: 2.04-8.09; p<0.0001) **(Figure 4A)** and stage III patients (HR = 3.01 [95% CI: 1.25-7.22]; p=0.014) **(Figure 4C).** In patients who did receive chemotherapy, low expression of KCNQ1 was associated with poor DFS in stage III patients (HR = 2.88 [95% CI: 1.44-5.77]; p=0.002) **(Figure 4D).** No association was observed between KCNQ1 expression and stage II patients who received adjuvant therapy **(Figure 4B)**, however, it should be noted that this subgroup of patients was relatively small (n=24). Collectively, these data indicate that loss of KCNQ1 expression is a strong prognostic biomarker to identify stage II and stage III colon cancer patients at high risk for disease recurrence, irrespective of adjuvant chemotherapy.

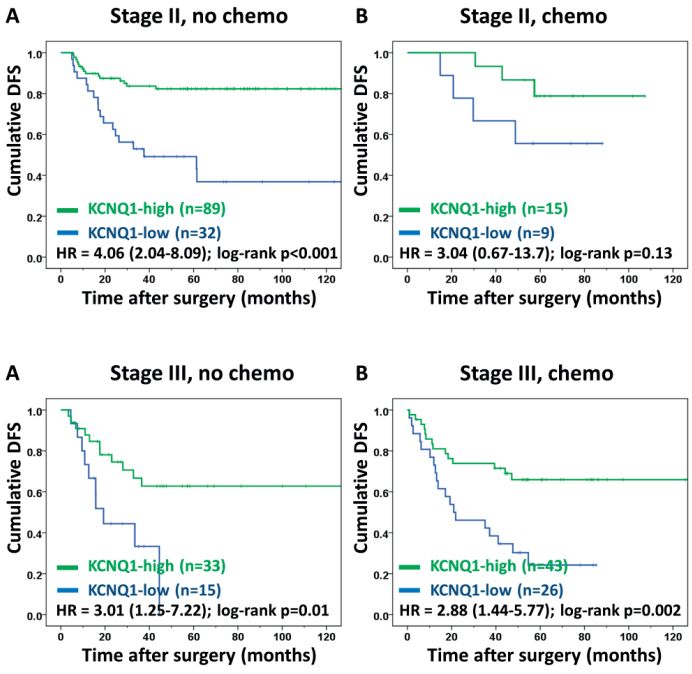

Figure 4: DFS curves for expression of KCNQ1 in MSS colon cancer patients not treated (A and C) and treated (B and D) with adjuvant chemotherapy for stage II (A and B) and stage III (C and D) disease. Cox regression HR (95% CI) and log-rank P-values are reported.

Loss of KCNQ1 is an independent prognostic factor in stage II and III colon cancer

In order to analyse the added value of KCNQ1 protein expression to current clinicopathological parameters, a multivariate model for 5-year DFS was built using stepwise backward Cox-regression. The variables 'isolated tumour deposits', 'nodal stage', 'angio-invasion', and 'MSI status' were retained in the model, in addition to KCNQ1 expression **(Table 2)**. These data demonstrate that loss of KCNQ1 expression is a strong predictor for poor DFS in the entire study population, with added value to established clinicopathological factors (HR = 3.75 [95% CI: 2.50-5.64]; p<0.0001) **(Table 2).**

KCNQ1 expression is correlated to expression of β-catenin and CD44 It has been postulated that expression of KCNQ1 is regulated by β-catenin and as such results from activation of the Wnt signalling pathway [26-28]. TMA's were stained for β-catenin and the Wnt-target gene CD44. Dichotomized expression was used to investigate whether KCNQ1 expression was correlated to active Wnt signalling. Indeed, KCNQ1 expression was significantly correlated to expression of β-catenin and CD44 in MSS colon cancers (Chi square test p=0.003 and p=0.001, respectively), but not in MSI colon cancers (p=0.076 and p=0.12, respectively). Despite these significant correlations, expression of nuclear β-catenin was not significantly associated with DFS (HR = 0.83 [95% CI: 0.58-1.21]; p=0.33) **(Figure 5A)**. In contrast, low expression of CD44 was associated with poor DFS (HR = 2.77 [95% CI: 1.35-5.68]; p=0.005) **(Figure 5B)**. However, the association of CD44 with DFS was less strong than that of KCNQ1, and was lost upon further analyses of subgroups of colon cancer patients based on stage, MSI status, or adjuvant treatment.

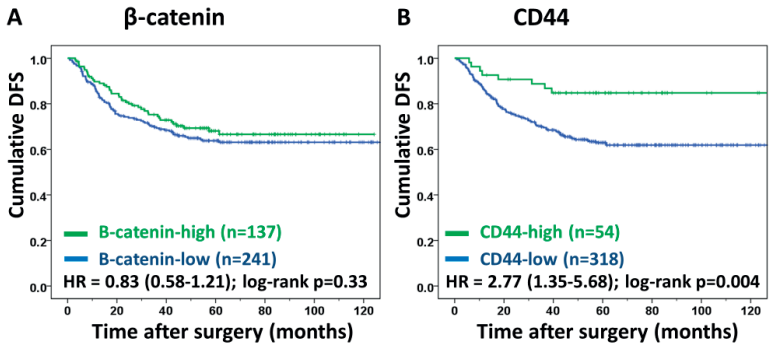

Figure 5: Association of DFS with expression of (A) nuclear b-catenin and (B) CD44. Cox regression HR (95% CI) and log-rank P-values are reported.

DISCUSSION

The present study addressed the prognostic value of KCNQ1 expression in stage II and stage III colon cancer patients. We here demonstrate that loss of expression of KCNQ1 in epithelial cells is strongly associated with a high risk for disease recurrence, and has additional prognostic value to clinicopathological features that are currently used to select high risk stage II patients such as angio-invasion, nodal stage, and MSI status. These findings are in line with our previous observation that KCNQ1 expression in CRC liver metastases was associated with poor overall survival [20]. Importantly, the prognostic value of KCNQ1 was clearly apparent in both stage II and stage III MSS colon cancers. In stage III tumours, its prognostic value was evident irrespective of treatment with adjuvant chemotherapy. Moreover, the prognostic value of KCNQ1 was also demonstrated in the smaller subgroup stage III MSI cancers. Only for stage II MSI tumours no significant association was observed between KCNQ1 and DFS, probably due to the limited number of patients in this subgroup combined with the fact that these patients have a relatively good prognosis [10,11]. Taken together, these data show that loss of KCNQ1 expression from neoplastic epithelial cells is a biomarker that strongly predicts disease recurrence and poor survival in stage II and III colon cancer.

At present, the most promising prognostic biomarkers for stage II colon cancer are based on mRNA signatures [13-19]. Making use of the external AMC-AJCCII-90 cohort from which one of these mRNA signatures was derived [14], we showed that low *KCNQ1* mRNA levels were associated with poor prognosis of stage II MSS patients. Protein expression of KCNQ1 was analysed in a second and independent cohort, which confirmed its prognostic value and demonstrated an even stronger association with DFS. Differences between *KCNQ1* mRNA and protein analysis may be due to the fact that mRNA levels are determined on tissue lysates, which include cells from both neoplastic epithelial origin as well as non-neoplastic cells in the tumour stromal compartments. In contrast, KCNQ1 protein analysis was focused on expression in neoplastic epithelial cells only, and its score is therefore not affected by variation in tumour stromal content. The hazard rate ratio for KCNQ1 protein expression in the present study (HR > 3.5 for stage II and HR > 4.0 for stage II MSS patients) was higher than that of biomarkers that have been identified previously using this study population, such as lamin A/C, AURKA, versican, lumican, Bcl-XL, FAS, Bcl-2 and FasL [29,34-36]. Only MGL ligand has been identified as a biomarker with comparatively strong effects in this study population, albeit restricted to the subgroup of stage III colon cancers [37]. Interestingly, the prognostic value of KCNQ1 protein expression in stage II CRC patients in the present study is comparable to or better than the hazard ratios reported for validated gene expression signatures like OncotypeDX Colon Cancer (HR's ranging from 1.38 – 1.96; [38-40], ColoPrint (HR's ranging from 2.16 – 2.65; [41-43], and GeneFx Colon (HR 2.53) [44]. Moreover, the protein staining-based KCNQ1 HRs exceed the mRNA signature-based HRs for differences between the poor survival CMS4 group and the CMS1-3 groups of colon cancers [15], implying that KCNQ1 may have a stronger prognostic value than the consensus-based mRNA signatures. While collection of frozen tissue for mRNA isolation is not common clinical practice, protein analysis by immunohistochemistry of FFPE material is. As such, implementation of KCNQ1 as a prognostic biomarker is technically well feasible in the current clinical setting.

Nuclear β-catenin and CD44 expression have commonly been used to indicate 'stemness' of colon cancer cells. Because β-catenin has been described to regulate KCNQ1 protein expression [26] and *KCNQ1* expression was shown to be increased upon activation of the Wnt signalling pathway [28], the correlation between KCNQ1 expression and nuclear β-catenin was examined, as well as

with the Wnt-target gene CD44. The current data demonstrated a positive correlation of KCNQ1 expression with both genes, and like for KCNQ1 also loss of CD44 expression was associated with poor DFS. In contrast, expression of nuclear β-catenin was not significantly associated with DFS. Therefore, while high Wnt-activity is thought to lead to development and progression of cancer, our data indicate that high expression of KCNQ1 was associated with better prognosis. As such, the present findings lend further support to previous observations in which silencing of several Wnt target genes by methylation stimulated tumour progression and was associated with poor prognosis [45,46]. This hypothesis is

further supported by the observation that loss of expression of KCNQ1 is significantly more prevalent in MSI tumours (54%) than in MSS tumours (31%). MSI is frequently caused by silencing of MLH1 in tumours with a CpG island methylator phenotype [47,48]. These data suggest that aberrant methylation is at least one mechanism that can cause silencing of *KCNQ1* expression, and show that loss of KCNQ1 expression plays a prominent role in the metastatic potential of both MSS and MSI cancers. Although KCNQ1 is not included in gene panels that are used to determine the CIMP phenotype in colorectal carcinomas [49], DNA hypermethylation of KCNQ1 is considered a hallmark of CIMP in renal cell cancer [50]. In the present study MSI tumors were overrepresented among right-sided tumors, consistent with literature [51,52]. Consequently, loss of KCNQ1 protein expression was also associated with right-sided tumors **(Table 1)**. In multivariate analysis, however, tumor location was not retained in the model while expression of KCNQ1 and MSI-status was **(Table 2)**."

Table 1: Clinicopathological characteristics of 377 colon cancer patients, stratified for low and high KCNQ1 protein expression. P-values were calculated by chi-square test or independent t-testing for continuous data. Significant p-values are printed in bold.

Clinicopathological characteristics	Total: n= 377 (%)	KCNQ1-low: N = 136 (%)	KCNQ1-high: N = 241 (%)	p-value
Sex				
Male	197 (52.3)	68 (50.0)	129 (53.5)	
Female	180 (47.7)	68 (50)	112 (46.5)	0.51
Age, median (range)(years)	72.9 (28.5-94.0)	72.7 (34.7-94.0)	73.2 (28.5-92.3)	0.80
Right sided tumour	170 (45.1)	73 (53.7)	97 (40.2)	**0.012**
Diameter, median (range)(mm)	40.0 (10-130)	40 (12-100)	35 (10-130)	0.09
Histological grade				
Well	24 (6.4)	9 (6.6)	15 (6.22)	
Moderate	296 (78.5)	97 (71.3)	199 (82.6)	
Poor	57 (15.1)	30 (22.1)	27 (11.2)	**0.017**
Stage				
II (=N_0)	223 (59.2)	76 (55.9)	147 (61.0)	
III (=N_+)	154 (40.8)	60 (44.1)	94 (39.0)	0.33
Tumour stage				
T1	4 (1.1)	1 (0.7)	3 (1.2)	
T2	18 (4.8)	5 (3.7)	13 (5.4)	
T3	318 (84. 3)	112 (82.4)	206 (85.5)	
T4	37 (9.8)	18 (13.2)	19 (7.9)	0.34
Nodal stage (stage III)				
N1	107 (28.4)	37 (27.2)	70 (29.0)	
N2	47 (12.5)	23 (16.9)	24 (10.0)	0.09
No. of lymph nodes, mean (s.d.)	8.9 (5.2)	8.7 (5.0)	9.0 (5.3)	0.57
Positive, mean (s.d.)	3.39 (3.3)	4.0 (4.0)	3.0 (2.8)	0.08
Mucinous differentiation.	80 (21.2)	44 (32.4)	36 (14.9)	**<0.001**
MSI-status				
MSI	63 (16.7)	34 (25.0)	29 (12.0)	
MSS	262 (69.5)	82 (60.3)	180 (74.7)	
Unknown	*52 (13.8)*	*20 (14.7)*	*32 (13.3)*	**0.001**
Ulceration	290 (76.9)	104 (76.5)	186 (77.2)	0.88
Angioinvasion	73 (19.4)	33 (24.3)	40 (16.6)	0.07
Emergency surgery	51 (13.5)	23 (16.9)	28 (11.6)	0.15
Perforation				
Before surgery	15 (4.0)	9 (6.6)	6 (2.5)	
During surgery	5 (1.3)	2 (1.5)	3 (1.2)	
After surgery	10 (2.7)	3 (2.2)	7 (2.9)	0.26
Tumour spill	12 (3.2)	5 (3.7)	7 (2.9)	0.68
Adjuvant chemo	122 (32.6)	46 (33.8)	76 (31.5)	0.65
Recurrence	123 (32.6)	74 (54.4)	49 (20.3)	**<0.001**
CRC mortality	98 (26.0)	58 (42.6)	40 (16.6)	**<0.001**
Overall mortality	173 (45.9)	76 (55.9)	97 (40.2)	**0.003**
Follow up, median (range) (months)	57.2 (2.8–148.6)	48.1 (2.8-129.2)	61.2 (4.14-148.6)	**<0.001**

Table 2: Multivariate backward Cox-regression analysis for disease free survival after 5 years of KCNQ1 and clinicopathological parameters that were retained in the model.

Parameters	HR	95%-CI	P-value
Isolated tumour deposits	1.594	0.98-2.59	0.06
Nodal stage	1.683	1.30-2.18	0.0001
Angio-invasion	2.062	1.32-3.22	0.001
MSI status	0.589	0.34-1.03	0.06
Low KCNQ1-expression	**3.752**	**2.50-5.64**	**<0.0001**

Functionally the *KCNQ1* gene encodes for a potassium channel protein. Potassium channels have an important role through their ion-channel function, with effects on proliferation, differentiation and apoptosis [53]. The active efflux of potassium changes its cellular concentrations with a subsequent passive shift of chloride regulated by *CFTR,* the gene affected in patients with cystic fibrosis. Transport of these ions causes the flow of water in and out of cells, thereby regulating cell volume which is known to have direct effects on apoptotic pathways in normal cells [54]. In this way, the function of ion-channels such as KCNQ1 and CFTR can affect cell survival [20,55]. Indeed, dysfunctional ion channels and cell volume regulation have been associated with resistance to apoptosis and resistance to chemotherapy [54]. However, the exact role of KCNQ1 amongst all K^+-channel proteins in this context is not known and therefore the relation of KCNQ1 expression to apoptosis and proliferation needs further elucidation. Furthermore, we previously demonstrated that *Kcnq1*-deficient tissues are enriched in *Cftr*-deficient gene signatures as well as in *Muc2*-deficient signatures [20]. The *Muc2* gene encodes for the main component of the protective mucus barrier Mucin2, which has a tumour suppressive role [56]. Disruption of normal CFTR function can also lead to a diminished mucus barrier that protects intestinal epithelial cells from bacterial contact [57]. These data suggest that loss of KCNQ1 can lead to excessive activation of innate immune signalling that may induce cancer-promoting inflammation.

In summary, we conclude that KCNQ1 is a strong prognostic biomarker for prediction of disease recurrence in stage II and III colon cancer patients. In particular, this biomarker could be very useful to stratify patients with stage II MSS for adjuvant therapy, since selection of patients who might benefit from such

treatment is still challenging. Further studies are needed both for prospective validation in a clinical setting and to address the functional role of *KCNQ1* in cell homeostasis, Wnt signalling and CRC development.

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CHAPTER 5

MACROD2 expression predicts response to 5-FU-based chemotherapy in stage III colon cancer

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ABSTRACT

Background

Colorectal cancer (CRC) is caused by genetic aberrations. *MACROD2* is commonly involved in somatic focal DNA copy number losses, in more than one-third of CRCs. In this study, we aimed to investigate the association of MACROD2 protein expression with clinical outcome in stage II and stage III colon cancer.

Methods

Tissue microarrays (TMA) containing formalin-fixed paraffin-embedded tissue cores from 386 clinically well-annotated primary stage II and III colon cancers were stained by immunohistochemistry and evaluated for MACROD2 protein expression. Disease-free survival (DFS) analysis was performed to estimate association with clinical outcome.

Results

Loss of nuclear MACROD2 protein expression in epithelial neoplastic cells of stage III microsatellite stable (MSS) colon cancers was associated with poor DFS within the subgroup of 59 patients who received 5-fluorouracil (5-FU)-based adjuvant chemotherapy (HR = 3.8 [95% CI: 1.4-10.0]; p=0.005).

Conclusion

These data indicate that low nuclear expression of MACROD2 is associated with poor prognosis of patients with stage III MSS primary colon cancer who were treated with 5-FU-based adjuvant chemotherapy.

INTRODUCTION

Colorectal cancer (CRC) has a worldwide incidence of over 1.3 million and is one of the leading causes of cancer-related deaths [1]. In the Western world, approximately one-third of CRC patients will die due to disease progression [2]. To estimate the prognosis of CRC patients, tumors are currently classified into stage I to IV according to the tumor-node-metastasis (TNM) staging, which is primarily based upon histopathological features of the tumor. Because somatic DNA alterations enable tumors to progress, characterization of genomic inter-tumor heterogeneity may reveal promising candidate biomarkers that could ultimately improve patient stratification for prognosis and therapy prediction. *MACROD2* has been shown to be commonly affected by focal deletions in CRC genomes [3-5], and has been identified to be the most frequently affected gene by structural variant (SV) breakpoints in CRC [5, 6]. The prevalence of chromosomal breakpoints in *MACROD2* is very high, *i.e.* 41% in a large series of 352 advanced CRC samples [6].

The function of MACROD2 is largely unknown. Recent studies demonstrated that MACROD2 is involved in highly dynamic mono-ADP-ribosylation (MARylation), which is a reversible post-translational protein modification that enables to control functions of target proteins. ADP ribose moieties can be attached to amino acid acceptor sites of target proteins by ADP-ribosyltransferases using the cofactor NAD+. Reversion of this modification is achieved by ADP-ribosylhydrolase activity. The macrodomain containing hydrolase MACROD2 can recognize mono-ADP-ribosyl groups and erase this motif from MARylated proteins. For example, the mono-ADP-ribosylhydrolase activity of MACROD2 is able to restore the WNT inhibitory function of the kinase GSK3B that is modified by PARP10-mediated MARylation [7-10]. Activation of WNT signaling is an important driver of CRC-development, and loss of MACROD2 function could thus contribute to CRC progression. Moreover, endogenous intracellular MACROD2 is recruited upon DNA damage and is able to reverse PARP1-mediated MARylation in the DNA-damage response [8].

In the present study, we examined the prognostic and predictive value of loss of MACROD2 protein expression in a series of 386 stage II and stage III clinically well-annotated primary colon cancers [11], and demonstrate that loss of

MACROD2 protein expression is associated with poor survival in the subset of stage III colon cancer patients who were treated with adjuvant 5-FU-based chemotherapy.

RESULTS

MACROD2 expression and disease recurrence

Prognostic value of MACROD2 protein expression was examined by evaluation of immunohistochemical staining on TMAs that contained tissue biopsies from 226 stage II and 160 stage III colon cancers. Intensity of nuclear MACROD2 protein expression of epithelial cells could be scored for 343 patients **(Figure 1)** while 25 stage II and 18 stage III cases could not be evaluated due to technical reasons such as loss of cores from TMA slides. Dichotomization of the scores resulted in 180 tumors with low (52%) and 163 tumors with high (48%) nuclear MACROD2 expression of neoplastic cells. Baseline clinicopathological characteristics of these patients in relation to MACROD2 expression are presented in **Table 1**. MACROD2-low colon cancers were associated with higher N-stage (p=0.03) **(Table 1)**.

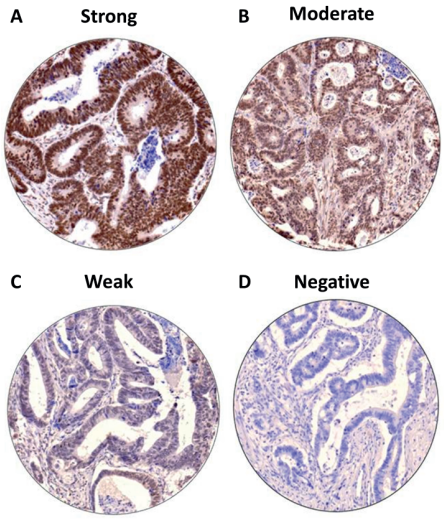

Figure 1: Representative examples of immunohistochemical staining intensities of MACROD2 expression, categories (A) 'strong', (B) 'moderate', (C) 'weak' and (D) 'negative', in stage II and III colon cancers.

Table 1: Baseline clinicopathological characteristics of 343 stage II and III colon cancer patients with MACROD2- low and MACROD2-high expression. Values in parentheses are percentages unless stated otherwise. P-values were calculated by chi-square tests or *t*-tests for continuous data. Bold p-values are considered significant (p<0.05).

Clinicopathological characteristics	Overall (n=343)	MACROD2-high (n=163)	MACROD2-low (n=180)	P-value
Sex				
Male	183 (53.4)	82 (50.3)	101 (56.1)	
Female	160 (46.6)	81 (49.7)	79 (43.9)	0.33
Age,				
Mean (s.d.)(years)	71.1 (11.9)	70.2 (13.1)	71.9 (10.7)	
Median (range)	73.2 (28.5-94.0)	73.9 (28.5-91.8)	72.8 (34.5-94.0)	0.20
Right sided tumour	152 (44.3)	69 (42.3)	83 (46.1)	0.55
Stage				
II	201 (58.6)	102 (62.6)	99 (55.0)	
III	142 (41.4)	61 (37.4)	81 (45.0)	0.19
Diameter, mean (s.d.)(mm)	41.5 (19.1)	40.6 (17.3)	42.3 (20.7)	0.45
Tumour stage				
T1	4 (1.2)	2 (1.2)	2 (1.1)	
T2	19 (5.5)	8 (4.9)	11 (6.1)	
T3	289 (84.3)	139 (85.3)	150 (83.3)	
T4	31 (9.0)	14 (8.6)	17 (9.4)	0.95
Nodal stage				
N0	201 (58.6)	102 (62.6)	99 (55.0)	
N1	97 (28.3)	48 (29.4)	49 (27.2)	
N2	45 (13.1)	13 (8.0)	32 (17.8)	**0.03**
No. of nodes examined, mean (s.d.)	9.0 (5.2)	8.8 (5.1)	9.2 (5.3)	0.50
Histological grade				
Well	20 (5.8)	10 (6.1)	10 (5.6)	
Moderate	274 (79.9)	128 (78.5)	146 (81.1)	
Poor	49 (14.3)	25 (15.3)	24 (13.3)	0.83
Mucinous differentiation.	67 (19.5)	38 (23.3)	29 (16.1)	0.12
Ulceration	262 (76.4)	125 (76.7)	137 (76.1)	1.0
Angioinvasion	65 (19.0)	26 (16.0)	39 (21.7)	0.23
MSI-status				
MSS	242 (70.6)	112 (68.7)	130 (72.2)	
MSI	56 (16.3)	30 (18.4)	26 (14.4)	
Unknown	45 (13.1)	21 (12.9)	24 (13.3)	0.40
Emergency surgery	46 (13.4)	23 (14.1)	23 (12.8)	0.84
Perforation				
Before surgery	15 (4.4)	6 (3.7)	9 (5.0)	
During surgery	5 (1.5)	0 (0.0)	5 (2.8)	
After surgery	10 (2.9)	4 (2.5)	6 (3.3)	0.15
Tumour spill	10 (2.9)	4 (2.5)	6 (3.3)	0.57
Adjuvant chemo	106 (30.9)	47 (28.8)	59 (32.8)	0.50
Recurrence	109 (31.8)	44 (27.0)	65 (36.1)	**0.09**
CRC mortality	86 (25.1)	36 (22.1)	50 (27.8)	0.28
Follow up, mean (s.d.) (months)	61.0 (33.2)	64.4 (33.3)	57.9 (32.9)	**0.07**

MACROD2 expression was not associated with disease-free survival (DFS) in stage II colon cancers **(Figure 2A).** In stage III colon cancers, however, low expression of MACROD2 showed a poorer DFS than high expression of MACROD2, although this difference did not reach statistical significance (HR = 1.6 [95% CI: 1.0-2.7]; p=0.07) **(Figure 2D).** Stratification by MSI status **(Table 1)** showed that in microsatellite stable (MSS) stage III colon cancers (n=109) low expression of MACROD2 was associated with poor DFS (HR = 2.0 [95% CI: 1.1-3.7]; p=0.02) **(Figure 2E).** This effect was not observed in MSS stage II colon cancers (n=133; p=0.9) **(Figure 2B).** The limited numbers of MSI stage II (n=33) and stage III (n=23) samples did not allow for meaningful comparison of DFS in MACROD2-low *versus* MACROD2-high MSI colon cancers **(Figure 2C, 2F).**

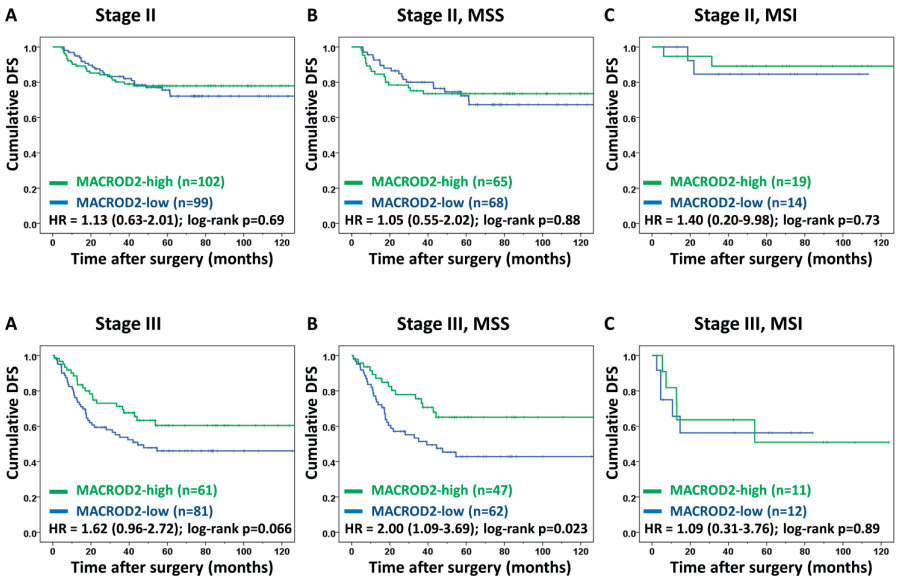

Figure 2: Kaplan-Meier plots for DFS (in months) stratified for MACROD2-high and MACROD2-low protein expression in stage II (A-C) and stage III (D-F) colon cancer patients including the subset of MSS (B, E) and MSI (C, F) patients. Log-rank p-values and Cox regression hazard ratios (HRs) with 95% confidence intervals (CIs) are reported.

MACROD2 expression and response to 5-FU-based adjuvant chemotherapy

Adjuvant chemotherapy could influence the prognostic effect of MACROD2 protein expression. Therefore, this parameter was used for further stratifica-

tion. In total 23 of 133 MSS stage II and 59 of 109 MSS stage III colon cancer patients were treated with 5-fluorouracil (5-FU)-based adjuvant chemotherapy. No effect of MACROD2 expression on DFS was observed within the subgroups that did not receive adjuvant chemotherapy **(Figure 3A, 3C).** However, loss of MACROD2 protein expression was strongly associated with poor DFS in MSS stage III colon cancer patients that did receive 5-FU-based adjuvant chemotherapy (HR = 3.8 [95% CI: 1.4-10.0]; p=0.005) **(Figure 3D).** The same tendency was observed for MSS stage II colon tumors, although the number of samples was too small to draw definitive conclusions

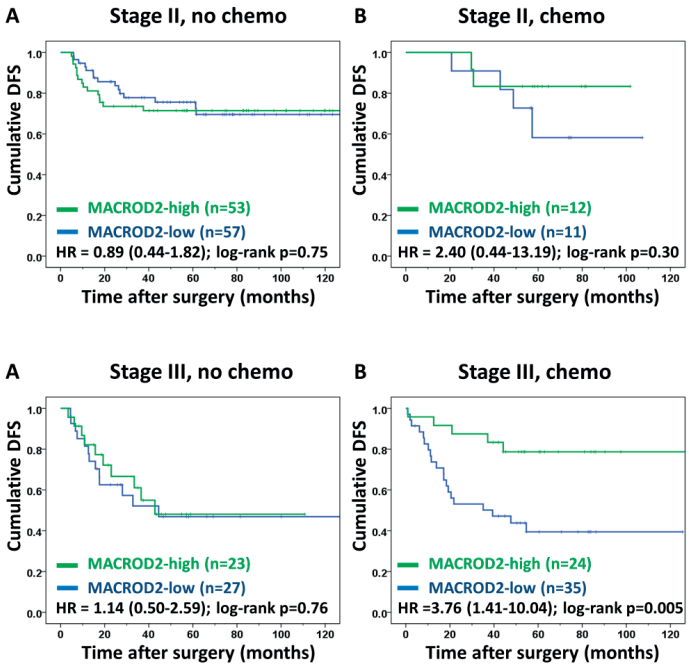

Figure 3: DFS curves (in months) for MACROD2 expression in MSS colon cancer patients who did not receive (A, C) and did receive (B, D) 5-FU-based adjuvant chemotherapy for stage II (A, B) and stage III (C, D) colon cancer patients. Log-rank p-values and Cox regression HRs (95% CI) are reported.

Multivariate analysis

Association of MACROD2 expression with DFS was tested by a multivariate model that included established clinicopathological parameters. This multivar-

iate model showed that MACROD2 expression was not an independent prognostic factor in the entire study population (data not shown). However, since MACROD2-low expression was associated with poor DFS in the subgroup of stage III colon cancer patients who received 5-FU-based adjuvant chemotherapy, two separate models were built for stage II and stage III colon cancers. While MACROD2 expression was not retained by the model in stage II colon cancers, MACROD2 expression was retained in the multivariate model for stage III colon cancers in addition to 'tumor location', 'T-stage', 'angioinvasion' and 'perforation' **(Table 2).**

Table 2: Clinicopathological parameters that were retained in a multivariate stepwise backward Cox-regression model ($p<0.05$) of stage III colon cancers

Parameters	HR	95%-CI	P-value
MACROD2 expression	**0.6**	**0.3-1.0**	**0.046**
Tumor location	1.8	1.0-3.1	0.041
T-stage	1.8	1.0-3.1	0.038
Angioinvasion	2.5	1.4-4.2	0.001
Perforation	1.4	1.0-2.0	0.042

DISCUSSION

The current study showed that low MACROD2 protein expression was associated with poor DFS in stage III MSS colon cancer patients who received 5-FU-based adjuvant chemotherapy **(Figure 3D).** The observation that MACROD2 expression was predictive in colon cancers treated for 5-FU-based adjuvant therapy **(Figure 3)** may suggest that the underlying biological mechanism is involvement of MACROD2 in DNA damage response, which is one of the known functions of MACROD2 [8]. It has previously been demonstrated that MACROD2 is involved in DNA damage signaling and is capable to reverse PARP1-mediated MARylation [8]. Notably, presence of MACROD2 could effectuate suppression of PARP1 activity [8], which is involved in DNA repair of incorporated 5-FU and its metabolites in the genome that exacerbates replication stress [12]. Consequently, tumor cells having low protein expression of MACROD2 may effectively enable PARP1-dependent DNA repair. Thus, one could speculate that 5-FU treatment combined with a small molecule PARP inhibitor may be lethal for tumor cells that have MACROD2-low protein expression. One *in vitro* study showed

that PARP inhibition synergizes with FdUrd, which is a metabolite of 5-FU, in MSI and MSS colon cancer cells [13].

MACROD2 has been identified to be the most frequent recurrent breakpoint gene in advanced CRC, which was observed in more than 40% of cases [6]. Accordingly, MACROD2 was a candidate biomarker to further examine its prognostic and predictive value. The present study demonstrated that low MACROD2 protein expression was associated with poor DFS, which is concordant with the hypothesis that SV breakpoints in *MACROD2* cause loss of normal gene function. However, we were not able to correlate SV breakpoints in *MACROD2* to loss of nuclear staining because all *MACROD2* breakpoints are located downstream of the first three exons that encode the epitope of the polyclonal antibody that was used for immunohistochemical staining (data not shown).

Although this study comprised a large retrospective cohort of 343 stage II and stage III colon cancer patients with well-documented clinical information, the sample size was insufficient to extensively test interactions of MACROD2 expression with other clinicopathological parameters. Furthermore, validation of the predictive value of MACROD2 expression for response to 5-FU-based therapy is required in a large independent prospective randomized clinical trial minimizing bias that might be introduced by unknown confounding factors associated with DFS rates and MACROD2 expression in the current study. In addition, as currently adjuvant treatment regimens are used other than 5-FU-based monotherapy, also the predictive effect of MACROD2 on 5-FU in combination with other chemotherapeutic agents such as irinotecan or oxaliplatin needs to be examined. It is unclear how MACROD2 expression may be associated with clinical outcome in relation to drug responsiveness, exemplified by a primary breast cancer study that showed that MACROD2 overexpression was associated with worse survival, probably due to resistance to anti-estrogen receptor-alpha therapy (tamoxifen) [14].

In conclusion, loss of nuclear MACROD2 protein expression predicts poor response to adjuvant 5-FU-based chemotherapy in MSS stage III colon cancers. Further studies are warranted to validate this potential biomarker to stratify colon cancer patients for response to 5-FU-based chemotherapy in the clinic

and to dissect the putative essential function of MACROD2 with respect to therapy resistance.

MATERIALS AND METHODS

MACROD2 immunohistochemistry using tissue microarrays

Archival formalin-fixed and paraffin-embedded (FFPE) material from 226 stage II and 160 stage III colon cancers was used to construct tissue microarrays (TMAs) as described by Belt *et al.* [11]. Microsatellite instability (MSI) status has previously been determined using a DNA-based test [11]. Tumor specimens and matched clinical data were obtained in compliance with the 'Code for Proper Secondary Use of Human Tissue in The Netherlands' https://www.federa.org/. A detailed overview of clinicopathological characteristics is given in **Table 1.**

Four µm sections of TMAs were mounted on glass slides, deparaffinized by xylene and rehydrated with a decreasing alcohol series. Staining for MACROD2 was performed upon antigen retrieval by microwave heating in citric acid (10 mM, pH6.0) and endogenous peroxidase neutralization in 0.3% hydrogen peroxide in methanol for 25 minutes. The primary rabbit polyclonal antibody directed against human MACROD2 (HPA049076; Atlas Antibodies AB, Stockholm, Sweden) was incubated one hour at a 1:175 dilution at room temperature, followed by incubation with polymer labeling for 30 minutes at room temperature (BrightVision, Immunologic, Duiven, The Nederlands). Secondary antibodies were visualized by liquid diaminobenzidine (DAB) substrate chromogen system. Slides were counterstained with Mayer's haematoxylin. Staining of FFPE normal kidney tissue was used as a positive control and incubation without primary antibody as a negative control.

Evaluation of MACROD2 protein expression

Immunohistochemical stainings were digitally captured as previously described [11]. Individual TMA core biopsies were scored for intensity of nuclear MACROD2 protein expression of neoplastic epithelial cells (categories: negative, weak, moderate, strong) **(Figure 1)** using dedicated TMA scoring software (v1.15.2, 3DHISTECH Ltd., Budapest, Hungary). TMA cores that contained less than 30% intact (epithelial) tumor tissue were considered non-representative

and excluded. TMA-cores from 56 tumors were evaluated by an independent observer (NTCvG) to assess inter-observer agreement for lowest MACROD2 intensity, which Cohen's weighted kappa score was K_w=0.6 [15, 16]. Intensity scores from tumor central and peripheral core biopsies [11, 17] were similar (Wilcoxon signed rank test, p=0.97).

Protein expression scores for MACROD2 were dichotomized for analysis of patient subgroups. First, the data was randomly split into five subsets. Next, the optimal cut-off for dichotomizing scores into a high- or low-expression group was based on 4/5th of the dataset using Receiver Operating Characteristic (ROC) curve analysis for survival data with 5-year DFS as the outcome of interest. This procedure was repeated five times, with 1/5th of the dataset varying. The final cut-off was the cut-off that was most often selected. In this way, the optimal cutoff for MACROD2 was set to 'low expression' for negative, weak and moderate intensity scores and 'high expression' for strong intensity scores. Optimal cutoff for MACROD2 was identical for all five iterations.

Statistical analysis

Statistical analysis was performed in R (version 3.2.2). Differences in baseline clinicopathological characteristics between patients with MACROD2- high and MACROD2-low protein expression were analyzed using Chi-square or student's *t*-tests. Univariate associations between DFS and MACROD2 protein expression was evaluated by Kaplan-Meier analysis. Cumulative survival rates were visualized by Kaplan- Meier curves (displayed for 120 months) and compared using a two-sided log-rank test (univariate). Hazard Ratios (HR) for MACROD2 expression were calculated using Cox regression analysis. Associations of DFS and known prognostic clinicopathological parameters were evaluated by multivariate Cox's proportional hazards regression analysis using stepwise backward elimination. Input parameters in addition to MACROD2 expression were tumor stage, T- and N-stage, isolated tumor deposits, MSI-status, tumor location (right sided), angioinvasion, histological grade, ulceration, perforation, and tumor spill [18-21]. This analysis was also performed by stratification for tumor stage. P-values less than 0.05 were considered significant.

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CHAPTER 6

KCNQ1 and lymphovascular invasion are key features in a prognostic classifier for stage II and III colon cancer.

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ABSTRACT

Background

The risk of recurrence after resection of a stage II or III colon cancer, and therefore qualification for adjuvant chemotherapy (ACT), is traditionally based on clinicopathological parameters. However, the parameters used in clinical practice are not able to accurately identify all patients with or without minimal residual disease. Some patients considered 'low-risk' do develop recurrence (undertreatment), whilst other patients receiving ACT might not have developed recurrence at all (overtreatment). We previously analysed tumour tissue expression of 28 protein biomarkers that might improve identification of patients at risk of recurrence. In the present study we aimed to build a prognostic classifier based on these 28 biomarkers and clinicopathological parameters.

Patient and methods

Classification and regression tree (CART) analysis was used to build a prognostic classifier based on a well described cohort of 386 patients with stage II and III colon cancer. Separate classifiers were built for patients who were or were not treated with ACT. Routine clinicopathological parameters and tumour tissue immunohistochemistry data were included, available for 28 proteins previously published. Classification trees were pruned until lowest misclassification error was obtained. Survival of the identified subgroups was analysed, and robustness of the selected CART variables was assessed by random forest analysis (1000 trees).

Results

In patients not treated with ACT, prognosis was estimated best based on expression of KCNQ1. Poor disease-free survival (DFS) was observed in those with loss of expression of KCNQ1 (HR = 3.38 (95% CI 2.12 – 5.40); p<0.001). In patients treated with ACT, key prognostic factors were lymphovascular invasion (LVI) and expression of KCNQ1. Patients with LVI showed poorest DFS, whilst patients without LVI and high expression of KCNQ1 showed most favourable survival (HR = 7.50 (95% CI 3.57 - 15.74); p<0.001). Patients without LVI and loss of expression of KCNQ1 had intermediate survival (HR = 3.91 (95% CI 1.76 – 8.72); p=0.001).

Conclusion

KCNQ1 and LVI were identified as key features in prognostic classifiers for disease-free survival in stage II and III colon cancer patients.

BACKGROUND

Colorectal cancer (CRC) is one of the most common types of cancer worldwide, with an incidence of nearly 2 million cases annually [1]. A global increase of CRC is foreseen, leading to over one million deaths in 2030. Nevertheless, survival itself has improved due to early detection, better diagnostics and improved treatment over the last years ([1-3]. These diagnostic- and treatment strategies nowadays result in 5-years disease-free survival rates of up to 85-90 % and 70-75% for stage II and stage III CRC, respectively [3, 4]. However, these are survival rates on a group level, and within both stages survival is different depending on T- and N-status. Furthermore, these survival rates are influenced by the administration of adjuvant chemotherapy (ACT) [5, 6]. All patients with stage III colon cancer have in fact an indication for ACT whereas stage II patients only qualify for ACT in case of high-risk features, which include T4, obstruction, perforation, vascular invasion and harvesting of limited lymph nodes. Besides stage and high-risk features, administration of ACT is subject to patient's fitness, age and post-operative complications. A reduction of risk of recurrence of 10-16 % has been assigned to this use of ACT in colon cancer [7-9]. This effect may be responsible for the relatively high DFS rate of up to 75% [4, 10-13]. However, there is also a subset of patients receiving futile treatment with ACT while suffering its side-effects. Taken together, making decisions who to offer adjuvant treatment based on tumour stage alone has significant limitations and is inadequate.

As many Western countries have implemented CRC-screening programs, a stage-shift is observed, reducing the number of patients who present with advanced cancer whilst increasing the proportion of patients who present with earlier stages of disease. Consequently, the question whether a patient is cured by surgery alone will become increasingly relevant in daily clinical practice, and requires better estimation of an individual's risk of disease recurrence. Several classifiers have been developed to better identify subgroups of colon cancer pa-

tients based on gene expression profiles, like the consensus molecular subtypes (CMS) [14]. However, despite the fact that one of these molecular subtypes was associated with poor prognosis, the diagnostic use of CMS classification has not reached clinical implementation yet.

In daily practice, clinical and pathological features are used to decide which individual patient qualifies for ACT. The pathological features are mainly based on routine immunohistochemical techniques that are widely used. Future prognostic biomarker assays based on immunohistochemistry may therefore be easily implemented in the routine diagnostics process. There is an ongoing quest to discover protein biomarkers that can be evaluated by immunohistochemistry with strong prognostic value, aiming to improve identification of patients with upcoming recurrence, such that ACT may be offered to those most likely to benefit. We previously evaluated tumour tissue expression of 28 proteins and identified multiple biomarkers with prognostic value, such as Aurora kinase A, Lamin A/C, CDX2, KCNQ1 and MACROD2 [15-20].

Despite progress made, it is still not possible to accurately identify all patients with or without upcoming recurrence. Therefore, the need for a prognostic classifier is deemed necessary to tailor treatment strategies in patients with stage II and stage III colon cancer. While we previously analysed many candidate biomarkers individually, the aim of the present study is to analyse what would be the optimal combination of biomarkers to determine prognosis and whether this combination of biomarkers outperforms individual biomarkers and routine diagnostics. Therefore, a prognostic classifier for DFS was built, based on routine clinicopathological parameters and tumour tissue expression data that was available from 28 previously published protein biomarkers.

PATIENTS AND METHODS

Study population and clinicopathological features

The study population comprised a well described retrospective cohort of 386 sporadic colon cancer patients. These stage II (n=226) and stage III (n=160) colon cancer patients had their primary surgical resection in the Spaarne Gasthuis (formerly Kennemer Gasthuis) hospital in the Netherlands. The as-

sessment for eligibility for the administration of ACT was based on guidelines available at the time. After surgery specimens were sent to the pathology lab for routine diagnostic workflows and subsequently stored in pathology archives. Clinical data, pathological parameters and archival tumour tissue material were collected in compliance with the 'Code for Proper Secondary Use of Human Tissue in The Netherlands' and conform local and national legislation that was applicable at the time [21]. This allowed us to perform the present retrospective observational translational research study without the additional need for study-specific informed consent from individual patients. For 332 patients MSI status was successfully determined previously [22], and was included as clinicopathological parameter. Whole tissue sections were evaluated by a dedicated pathologist for evaluation of LVI, defined as presence of tumour cells within the lumen of lymph vessels, on D2-40 or hematoxylin-eosin stained sections.

Biomarker features

We previously evaluated 28 protein biomarkers by scoring immunohistochemical stainings of tissue micro arrays (TMA), as described previously [15-20, 23-25]. Details on immunohistochemical staining, scoring, dichotomization and univariate results of these 28 markers are described in **Supplementary Table 1** (appendices, chapter 9). A brief summary of our biomarker workflow is presented in our **Supplementary method 1** (appendices, chapter 9).

Patient subsets

The cohort contained patients who were not treated with 5FU-based adjuvant chemotherapy (ACT) (n= 263) and who were treated with ACT (n= 123). Some biomarkers might have prognostic value (risk of disease recurrence) and/or predictive value (responsiveness to ACT). Because it is not possible to distinguish prognostic from predictive impact of biomarkers in patients treated with ACT, CART analyses were performed separately for patients who were and those who were not treated with ACT.

CART and statistical analysis

Dichotomized data of 28 protein biomarkers were used combined with all available clinical and pathological parameters. Differences in clinical and pathological variables between groups treated with and without ACT were analysed

using the chi-square and Mann Whitney U test. For both treatment groups, classification and regression tree (CART) analysis for survival data was performed, aiming to select the best prognostic subset of parameters. CART is a nonparametric approach and therefore does not assume that the data originates from a particular parametric distribution. Furthermore, the CART algorithm incorporates both model fitting and cross-validation to avoid overfitting the model. CART can use the same variables more than once in different parts of the tree: this capability can uncover complex interdependencies or synergies between sets of variables. Endpoint was disease-free survival (DFS), defined as time from surgery to recurrent disease (in months). Minimum number of observations per node was set at 50, and trees were post-pruned by trimming the tree in a bottom-up fashion, until a tree remained with the lowest misclassification error rate [26]. Patients without expression scores for a certain biomarker were allocated based on the 'logical leaf', based on distribution of the available expression scores. To assess robustness of the selection of markers in the pruned CARTs, random forests analysis with 1000 trees was performed. Ranking of importance of all markers was obtained. Both CART and Random Forest analysis were performed using RStudio, for which the script is presented in **Supplementary Method 2** (appendices, chapter 9). Subgroups as defined by this CART analysis were used for further statistical evaluation. DFS of these subgroups was visualized by Kaplan–Meier curves, and p-values were obtained from log-rank tests. Hazard ratio (HR) with 95% confidence interval (CI) were calculated using Cox regression analysis. Missing values were excluded for survival analysis. Statistical analyses were performed with IBM SPSS Statistics. The workflow of this study was summarized in **Figure 1.**

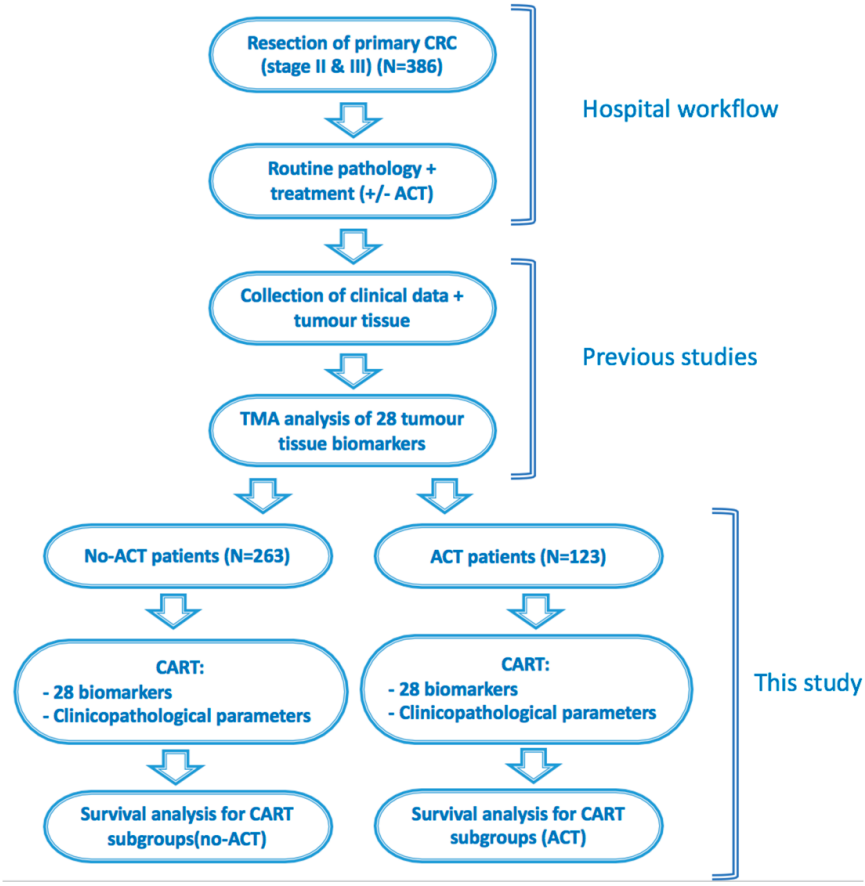

Figure 1: Workflow of this study. All protein biomarker studies on this cohort were based on 386 patients with stage II and III colon cancer. After primary resection, and without disturbing clinical workflows (i.e. diagnostics and the decision of whether or not adjuvant chemotherapy (ACT) was administered), clinical data and tumour tissues were obtained. These tissues were previously analysed for 28 promising biomarkers. These results and routine clinicopathological parameters were included in this CART analysis, with separate analysis for patients not treated and treated with ACT. For each treatment group the classifier was subsequently associated with disease-free survival.

RESULTS

Of the 386 patients with stage II and III colon cancer included in this cohort, availability of protein expression scores for individual biomarkers ranged from 334 to 384. Median follow up of this cohort was 57,2 months. Recurrence rate

was 29.3% and 40.7 % (p=0.036) for patients treated without and with ACT, respectively. Furthermore, the group of patients treated with ACT was younger compared to the untreated group (64.9 vs 76.3 year; p<0.001), and included more stage III patients. These, and other baseline clinicopathological parameters included in this CART analysis, stratified for ACT, are shown in **Table 1**.

Table 1: Baseline parameters of this cohort of 386 stage II and III colon cancer patients, stratified for ACT (P-values for differences in baseline between ACT treated and untreated patients were calculated using chi-square, or Mann Whitney U when appropriate).

Clinicopathological characteristics	**Total: n= 386 (%)**	**No ACT: N = 263 (%)**	**ACT: N = 123 (%)**	**P-value**
Sex				
Male	203 (52.6)	127 (48.3)	76 (61.8)	
Female	183 (47.4)	136 (51.7)	47 (38.2)	**0.016**
Age, median (range)(yr)	73.1 (28,5 – 94.0)	76.3 (28.5 – 94.0)	64.9 (34.5 – 83.3)	**<0.001**
Right sided tumour	173 (45.1)	117 (44.5)	56 (45.5)	0.91
Diameter, median (range)(mm)	40.0 (10.0 – 130.0)	40.0 (10.0 – 130.0)	35.0 (10.0 – 100.0)	**0.009**
Histological grade				
Well	24 (6.2)	17 (6.5)	7 (5.7)	
Moderate	302 (78.2)	206 (78.3)	96 (78.0)	
Poor	60 (15.5)	40 (15.2)	20 (16.3)	0.93
Stage				
II (=N_0)	226 (58.5)	192 (73.0)	34 (27.6)	
III (=N_+)	160 (41.5)	71 (27.0)	89 (72.4)	**< 0.001**
Tumour stage				
T1	4 (1.0)	2 (0.8)	2 (1.6)	
T2	19 (4.9)	8 (3.0)	11 (8.9)	
T3	325 (84.2)	231 (87.8)	94 (76.4)	
T4	38 (9.8)	22 (8.4)	16 (13.0)	**0.022**
Nodal stage (stage III)				
N1	110 (28.5)	54 (20.5)	56 (45.5)	
N2	49 (12.7)	17 (6.5)	32 (26.0)	**<0.001**
Mucinous differentiation.	82 (21.2)	64 (24.3)	18 (14.6)	**0.033**
Isolated tumour deposits (ITD)	50 (13.0)	24 (9.1)	26 (21.1)	**0.001**
MSI-status				
MSI	65 (16.8)	47 (21.4)	18 (16.1)	
MSS	332 (86.0)	173 (78.6)	94 (83.9)	0.31
Ulceration	297 (76.9)	196 (74.5)	101 (82.1)	0.12
Lymphovascular invasion	78 (20.2)	44 (16.7)	34 (27.6)	**0.015**
Emergency surgery	51 (13.2)	35 (13.3)	16 (13.0)	1.0
Perforation				
Before surgery	16 (4.1)	12 (4.6)	4 (3.3)	
During surgery	5 (1.3)	4 (1.5)	1 (0.8)	
After surgery	10 (2.7)	9 (3.4)	1 (0.8)	0.38

Table 1: *(Continued)*

Clinicopathological characteristics	Total: n= 386 (%)	No ACT: N = 263 (%)	ACT: N = 123 (%)	P-value
Tumour spill	12 (3.1)	9 (3.4)	3 (2.4)	0.76
Recurrence	127 (32.9)	77 (29.3)	50 (40.7)	**0.036**
CRC mortality	101 (26.2)	64 (24.3)	37 (30.1)	0.26
Overall mortality	177 (45.9)	133 (50.6)	44 (35.8)	**0.008**
Follow-up (median, range) (months)	57,2 (3 – 148)	57.1 (3 – 148)	57.5 (3 – 127)	0.73

CART analysis of patients not treated with ACT

For ACT-untreated patients (n=263) the pruned tree with lowest misclassification error rate consisted of one node only, i.e. KCNQ1 **(Figure 2a).** The first leaf was defined as KCNQ1-low (n=94) with 46 recurrences (48.9%). The second leaf was defined as KCNQ1-high (n=169) with 31 recurrences (18.3%). Cox regression analysis based on this stratification, showed that the subgroup with loss of expression of KCNQ1 was significantly associated with poor survival (HR = 3.38 (95% CI 2.12 – 5.40); p<0.001; **Figure 2b).** The original unpruned tree is shown in **Supplementary Figure 1** (appendices, chapter 9)**.**

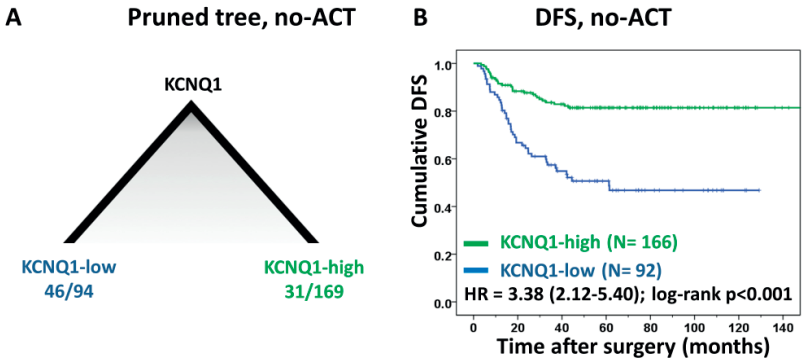

Figure 2: A. Pruned tree based on CART analysis for patients not treated with ACT, showing two distinct classes based on expression of KCNQ1, with the number of events (recurrence) and the total number of patients in each class. B. Disease-free survival (DFS) for patients not treated with ACT, visualised by Kaplan Meier curves and stratified for KCNQ1-low and KCNQ1-high. Cox regression HR (95% CI) and P-values are reported.

CART analysis of patients treated with ACT

For ACT-treated patients (n=123) the pruned tree consisted of 2 nodes, i.e. LVI and KCNQ1 **(Figure 3a)**. The first leaf consisted of patients with LVI (n=34) with 25 recurrences (73.5%). The second leaf consisted of patients without LVI that were KCNQ1-low (n=27), with 15 recurrences (55.6%). The third consisted of patients without LVI that were KCNQ1-high (n=62), with 10 recurrences (16.1%). Cox regression analysis and Kaplan Meier curves showed that DFS was significantly different between these three subgroups. Patients with LVI showed poorest prognosis, whilst patients without LVI but with high expression of KCNQ1 showed most favourable prognosis (HR = 7.50 (95% CI 3.57 - 15.74); p<0.001). Patients without LVI and with loss of expression of KCNQ1 had intermediate prognosis compared to the most favorable subgroup (HR = 3.91 (95% CI 1.76 – 8.72); p=0.001; **Figure 3b)**. The original unpruned tree is shown in **Supplementary Figure 2** (appendices, chapter 9).

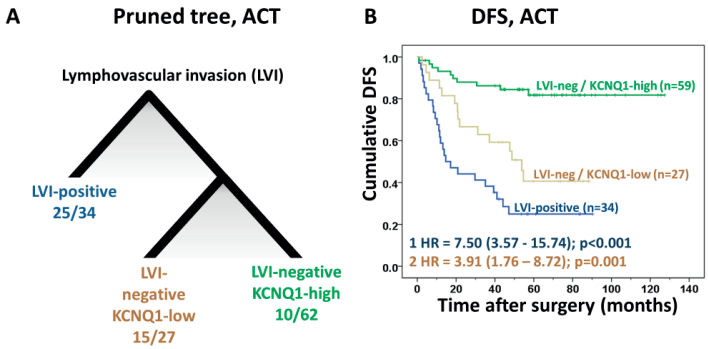

Figure 3: A. Pruned tree based on CART analysis for ACT-treated patients, showing three distinct classes based on lymphovascular invasion (LVI) and expression of KCNQ1, with the number of events (recurrence) and the total number of patients in each class. B. Disease-free survival (DFS) for patients treated with ACT, visualised by Kaplan Meier curves and stratified for the three subgroups of the CART analysis. Cox regression HR (95% CI) and P-values are reported, with LVI-negative/KCNQ1-high (green) as reference category.

Random forest analysis and variable importance

The ranking of importance of all features is shown in **Supplementary Figure 3** (appendices, chapter 9). Variable importance was defined as the proportion

of times a variable is selected in the fitted trees within a random forest, and visualized by variable importance graphs. For the patients not treated with ACT, KCNQ1was the most important feature. For the ACT-treated patients LVI, KCNQ1 and MACROD2 were key features.

DISCUSSION

In this study, we aimed for a classifier based on a minimal set of complementary markers with maximal prognostic value for stage II and III colon cancer. For patients not treated with ACT KCNQ1 was the single best marker to stratify for, with survival benefit for patients with high expression of KCNQ1. For ACT-treated patients stratification for LVI at first, followed by expression of KCNQ1 for the LVI-negative tumours, was most informative. Tumours with LVI were associated with poor survival. For the subset of tumours without LVI, high expression of KCNQ1 was subsequently associated with the best survival.

The fact that KNCQ1 and LVI were the main and only prognostic features in the pruned CART analyses implies that, following stratification for these features, there were no further CRC subgroups for which any of the other protein biomarkers could provide additional prognostic information. These features were even stronger than tumour stage and MSI-status, which are known to have relatively strong prognostic value and were each included as variables in the CART analysis. Instead, MACROD2 was a feature of similar importance as KCNQ1 and LVI in the group of patients treated with ACT (**Supplementary Figure 3,** appendices, chapter 9). Previous analysis showed that MACROD2 was a predictive biomarker for response to ACT in stage III microsatellite stable (MSS) patients [20] and as such its potential relevance in ACT-treated patients was in line with our expectations.

The results of these classifiers emphasize that KCNQ1 was identified as a strong prognostic biomarker for disease recurrence in both stage II and III colon cancer patients, irrespective of MSI-status and/or treatment with ACT [16]. KCNQ1 encodes an ion channel protein, which acts both as a target gene and regulator of the Wnt/β-catenin pathway [27, 28]. Loss of KCNQ1 is associated with poor prognosis, CRC cell proliferation, epithelial-to-mesenchymal transition and tu-

morigenesis [18, 27, 28]. In this study, loss of KCNQ1 protein expression appears to be the most informative prognostic feature among 28 promising biomarkers and routine clinicopathological parameters, together with LVI in ACT-treated patients.

LVI is also known as a strong prognostic factor associated with more aggressive tumour behaviour and poor prognosis [29-31]. In theory, all stage III patients are expected to have LVI to some degree because LVI is required to enable lymphatic spreading of tumour cells, and thus progression from stage II to stage III CRC [32]. However, tumours with LVI may not show this feature in every tissue-section that is evaluated by a pathologist, which explains the apparent discrepancy between the amount of stage III (N=160) and LVI-positive (N=78) tumours, at a ratio that is also observed for other patient cohorts [33]. Vascular invasion, and especially extramural vascular invasion, is also associated with poor prognosis, but is less common than LVI and beyond the scope of this study [34, 35].

The present study indicates that determination of KCNQ1 protein expression in patients not treated with ACT, and LVI-status combined with KCNQ1 expression in patients treated with ACT is currently the optimal approach to determine prognosis with a minimal number of key prognostic features. In particular for the group of patients treated with ACT the HRs observed for the combined LVI and KCNQ1 analysis (HR = 3.9 and HR = 7.5; **Figure 3B**) exceeded the univariate HRs of LVI (HR = 3.5) and KCNQ1 (HR = 3.3), respectively (data not shown). Whether this is just informative for prognosis, or that it might help the selection of patients who would benefit from ACT, remains to be validated in an independent cohort. Moreover, combining tumour tissue analysis with other techniques to identify high-risk tumours, like measuring post-surgical liquid biopsy cell-free circulating tumour DNA as a marker for minimal residual disease, may further enhance prognostic value of tumour tissue-based classifiers [36, 37].

CONCLUSION

KCNQ1 and lymphovascular invasion were identified as key features in classifiers for prognosis in stage II and III colon cancer patients, either not treated or treated with ACT. Although this classifier was not able to create a prediction

model for future patients yet, it reinforced the prognostic value of KCNQ1 and lymphovascular invasion, and the need to prospectively evaluate (the combination of) these biomarkers in future studies.

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CHAPTER 7

Summary, discussion and future perspectives

1. SUMMARY

As we learn more and more about the underlying biology of colon cancer, biomarkers will play a prominent role in the improvement of the identification of patients with high risk of recurrence. Once the identification of the prognostic tumour characteristics has been optimized an appropriate therapy can be advised, whereas others may safely choose to refrain from adjuvant therapy. In this thesis we tried to optimize the process of identification of patients with poor prognosis, the efficacy, the safety and the cost-effectiveness of adjuvant therapies need to be addressed separately, and was beyond the scope of this thesis.

In **Chapter 2** we described the prognostic value of microvessel density (MVD) as a surrogate marker for angiogenesis, one of the hallmarks of cancer. As angiogenesis itself remains difficult to measure directly, MVD may provide insight in this microenvironmental process. This computerized morphological study showed that MVD increases with stage, which may explain the observation that stage II patients with high MVD had poor prognosis, biologically being a kind of pre-stage III. Actual stage III patients with high MVD, that were all treated with adjuvant 5-FU based chemotherapy, showed better prognosis on the contrary. We hypothesized that residual or recurrent tumour tissue in stage III patients, from primary tumours with high MVD, were better penetrable for chemotherapy, explaining the improved survival.

In **Chapter 3** we tried to validate CDX2 immunohistochemically, being a promising biomarker published in a high-impact journal by Dalerba et al. Our study showed the practical hurdles involved in validating biomarkers in other cohorts. For disease free survival we were not able to find significant differences between high and low expression of CDX2. However, for disease specific survival we did find comparable results. For a subset of 41 patients we analysed expression of CDX2 by both immunohistochemistry and mass spectrometry. Interestingly, discriminatory power of CDX2 as a prognostic biomarker detected by mass spectrometry outperformed the immunohistochemical detection method. This observation shows that in some cases the biomarker itself may be promising, but the method ought to be fine-tuned to benefit from that marker's full potential.

In **chapter 4** we presented a new biomarker, obtained from promising research in mice. Expression of KCNQ1 and CD44, both regulated by Wnt-signalling, was analysed immunohistochemically in 386 stage II and III patients, and on mRNA in an external cohort of 90 patients. We concluded that KCNQ1 was a strong prognostic biomarker for disease recurrence. KCNQ1 may be particularly useful in stage II MSS patients, where the question remains which patients are at risk for recurrence and might benefit from adjuvant chemotherapy.

In **Chapter 5** MACROD2 was discussed, a relatively unknown gene, although a tumour suppressing function by activating PARP1 and DNA repair is suspected. We indeed found that low nuclear expression was associated with poor prognosis in stage III MSS colon cancer patients, treated with 5-FU based ACT. Even more, high expression of MACROD2 may serve as a predictive biomarker in stage III MSS tumours, favouring adjuvant treatment with 5-FU compared to no adjuvant treatment at all.

In the final chapter, **Chapter 6,** we describe an ultimate attempt to improve the identification of high risk patients. Several biomarkers have been studied on our cohort over the years, some with promising and some with disappointing results. For this study we intended to analyse all these markers combined, to find out which (combination of) biomarkers was able to estimate prognosis best, and whether or not previous less important biomarkers became more interesting. Although other techniques were also used on (some subsets of) this cohort, i.e. mass spectrometry, next generation sequencing and morphological analysis (MVD), for this study we chose for a feasible and widely applicable technique like immunohistochemistry. Therefore, all immunohistochemical biomarkers examined previously on tissue micro arrays were included, in addition to all common clinical and pathological parameters available in our cohort. These were all included in a Classification and Regression Tree (CART) analysis, which showed that both lymphovascular invasions (LVI) and KCNQ1 were the key features for estimating prognosis in stage II and III colon cancer.

2. DISCUSSION AND FUTURE PERSPECTIVES

In an ideal world, prognosis could be estimated accurately using an easy and widely available biomarker assay, like immunohistochemistry. This ideal biomarker should represent a single important function, or as a derivative for a key biological process. Alternatively, as time and technical development progress, more quantative assays might be needed to take advantage of the full prognostic potential of a biomarker. Hypothetically, already promising immunohistochemical biomarkers might even have better results when such assays were used. However, on the down-side, it would make them less applicable for widespread use (inter)nationally because these techniques might not be available in every hospital, and certainly not in all countries around the world. Another advantage of immunohistochemistry is the ability to correct for spatial distribution of a biomarker, i.e. by scoring only epithelium, nuclear or cytoplasmic expression, or stroma. For more quantative assays like mass spectrometry this distinguishment can often not be made, since whole tumour tissues are used and one cannot distinguish whether or not expression was found in stroma or epithelium. This problem can be overcome when the biomarker of interest has proven to only have epithelial expression, in that case a more quantative assay might be as 'spatially responsible' as immunohistochemistry.

The main patient population on which this thesis is based is of reasonable size, and although included relatively a long time ago, it is still representative for western colon cancer populations. Furthermore, the cohort was well defined over the years and data and tissues were stored properly for additional studies. An older version of the TNM (4th edition) was used for including stage II and III colon cancer patients, however newer versions would not have led to inclusion in other subgroups. Difference between the 4th and the 8th editions is the split-up of stage 4 into T4a and T4b, and those patients are not included in this thesis. Furthermore, in theory some stage T1-2N0M0 patients in older TNM-versions would have been included as stage IIIa nowadays when the N1c-status was present. However, again these patients, being stage I, were not included in this cohort. Within this cohort, 19 of the 226 stage II tumours (T3-4N0/M0) with isolated tumour deposits (ITD) would nowadays have been included as stage III instead of stage II, using the most recent TNM-version. No significant dif-

ferences in recurrence or mortality were found between the ITD-positive and ITD-negative stage II tumours.

Putting all of our results in perspective, one may notice that both the results of CDX2 (measured by mass spectrometry) and the CART analysis stand out from the others with regard to hazard ratio's (HR). Univariate HR's for our immunohistochemical biomarkers reach up to HR's of 4,0 for specified subgroups. One could hypothesize that analysis of for instance KCNQ1, CD44 or MACROD2 by other more quantative modalities than immunohistochemistry could have led to even higher HR's. Although this should not be taken as a plea for routinely writing-off immunohistochemistry, more quantative analyses for the right biomarker might help implementation into clinical practice. Therefore, one should embrace the possibility that both marker and modality matter, and that sometimes combining rather than only discovering new biomarkers deserves attention.

In this light, several strategies are possible for future studies. On one hand, one could focus on discovering new biomarkers, regardless of which technique is used. On the other hand, efforts can be undertaken to re-evaluate promising markers, regardless of their immunohistochemical results, with more quantative assays. For instance, KCNQ1 could be analysed by a more quantative assay like mass spectrometry, or for instance a larger cohort (than the 90 patient described in chapter 4) for mRNA. Alternatively, MicroRNA's may prove useful in the future, as new biomarkers or as surrogate markers for known pathways or biomarkers.

As post-transcriptional regulators of expression of several oncogenes and tumour suppressor genes, they have demonstrated to be able to mediate signalling pathways leading/contributing to cancer. Since MiRNA's are relatively stable, they can be detected in biological fluids and archival tissues [1].

Another study that might contribute to the insight in the tumour microenvironment, structurally, is analysis of lymph vessel density (LVD). In line of analysis of microvessel density with CD31, one could digitally capture lymph vessels as well, and analyse whether or not the density of lymph vessels has prognostic value. These lymph vessels could be immunohistochemically stained with D2-40, a marker that shows staining in lymphatic channel endothelium, but not

in the adjacent capillary. Analysing LVD in addition to MVD might enhance the insight in the structural lay-out of the tumour, and this might improve prognostic potential of MVD (or LVD) alone, potentially in addition to lymph angioinvasion (LVI). Furthermore, classifiers or panels of immunohistochemical markers are of interest, since that may lead to stronger results than the sum of its parts. Besides the question who to treat with adjuvant chemotherapy after surgical resection, a biomarker may in the future also answer the question who needs a stricter follow up scheme? And who might be put on a more liberal one? This may also influence clinical practice and health care costs in the future.

Another promising approach is the identification of minimal residual disease (MRD) by analysing the presence of circulating tumour DNA (ctDNA) after surgery. Presence of ctDNA after surgery is associated with a high risk of recurrence. It would be interesting to analyse if microvessel density (MVD) or lymphangioinvasion (LVI) can be associated with MRD, since one can hypothesize that these are the tumours that offer more 'access' to the bloodstream. If this association can be found, detection of ctDNA in patients with low MVD might not be as useful, or results of ctDNA studies would improve when MVD-high and –low patients are analysed separately. Recently, it was stated that ctDNA might guide adjuvant treatment of stage II colon cancer patients, leading to less stage II patients treated with adjuvant chemotherapy without a reduction in recurrence free survival [2]. Combining this concept of ctDNA with strong immunohistochemical biomarkers may further improve accurate selection of stage II patients at risk of recurrence. On the other hand, negative-ctDNA stage III cancers with an advantageous immunohistochemical profile might be treated without ACT, but with rigorous follow up only. This advantageous profile could consist of KCNQ1 and lymphangioinvasion, or for instance a combination of microvessel density and lymph vessel density.

Although we have found or validated very promising biomarkers, the author realizes that these will not have immediate effect on clinical practice. However, hopefully these studies contribute to the evidence for biomarkers such as KCNQ1 and CDX2, and form the basis for further research. Further research should focus on the steps of the roadmap to biomarker validation, i.e. assay development and (prospective) validation of both marker and technique. Sub-

sequently, before the (ideal set of) biomarkers are approved and implemented, cost effectiveness analysis and maybe even clinical trials are needed.

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CHAPTER 8

Nederlandse samenvatting

NEDERLANDSE SAMENVATTING

De resultaten van dit proefschrift dragen bij aan het inzicht in de prognose van stadium II en III coloncarcinoom. Naarmate we meer leren over de onderliggende tumor biologie, zullen er steeds meer biomarkers ontdekt worden, en zullen deze een prominente(re) rol spelen bij de verbetering van de identificatie van patiënten met een hoog risico op een recidief. Zodra de identificatie van een tumor met een slechte prognose is geoptimaliseerd, kan voor de juiste patiënt de geschikte therapie worden geadviseerd, en anderen kunnen er juist veilig voor kiezen om de aanvullende chemotherapie achterwege te laten. In dit proefschrift hebben we geprobeerd het proces van identificatie van patiënten met een slechte prognose te optimaliseren, terwijl de werkzaamheid, de veiligheid en de kosteneffectiviteit van adjuvante therapieën afzonderlijk moeten worden onderzocht: dit viel buiten het bestek van dit proefschrift.

In Hoofdstuk 2 hebben we de prognostische waarde van microvessel density (MVD) beschreven als een surrogaatmarker voor angiogenese, een van de belangrijke biologische kenmerken van kanker. Omdat angiogenese zelf moeilijk direct meetbaar is, kan MVD inzicht verschaffen in dit proces. Deze gecomputeriseerde morfologische studie toonde aan dat MVD toeneemt met het stadium, wat kan verklaren dat stadium II-patiënten met een hoge MVD een slechtere prognose hadden, biologisch gezien als een soort pre-stadium III. Werkelijke stadium III-patiënten met een hoge MVD, die allemaal werden behandeld met adjuvante chemotherapie op basis van 5-FU, vertoonden juist een betere prognose. We veronderstelden daarom dat resterend of terugkerend tumorweefsel in stadium III patiënten, afkomstig van primaire tumoren met een hoge MVD, beter doordringbaar was voor chemotherapie, wat de verbeterde overleving zou kunnen verklaren.

In Hoofdstuk 3 hebben we geprobeerd om CDX2 immunohistochemisch te valideren, een veelbelovende biomarker die is gepubliceerd in een high-impact tijdschrift door Dalerba et al. Onze studie toonde onder meer de praktische hindernissen die betrokken zijn bij het valideren van biomarkers in een nieuw cohort. Voor ziektevrije overleving (disease free survival, DFS) waren we niet in staat om significante verschillen te vinden tussen hoge en lage expressie van CDX2. Voor ziekte specifieke overleving (disease specific survival, DSS)

vonden we echter vergelijkbare resultaten. Voor een subset van 41 patiënten analyseerden we de expressie van CDX2 met behulp van zowel immunohistochemie als ook massaspectrometrie. Hieruit bleek interessant genoeg dat het onderscheidende vermogen van CDX2 als een prognostische biomarker gedetecteerd door massaspectrometrie veel beter presteerde dan met behulp van de immunohistochemische detectiemethode. Deze observatie toont aan dat de biomarker zelf in sommige gevallen veelbelovend kan zijn, maar dat de methode soms verfijnd moet worden om te profiteren van het volledige potentieel van die marker.

In hoofdstuk 4 hebben we een nieuwe biomarker gepresenteerd, verkregen uit veelbelovend onderzoek bij muizen. Expressie van KCNQ1 en CD44, beide gereguleerd door Wnt-signalering, werd immunohistochemisch geanalyseerd bij 386 stadium II en III patiënten, en op mRNA in een extern cohort van 90 patiënten. We concludeerden dat KCNQ1 een sterke prognostische biomarker was voor terugkeer van de ziekte. KCNQ1 kan met name nuttig zijn bij stadium II MSS-patiënten, waarbij de vraag blijft welke patiënten een risico lopen op een recidief, en dus baat kunnen hebben bij adjuvante (chemo)therapie.

In Hoofdstuk 5 werd MACROD2 besproken, een relatief onbekend gen, hoewel een tumor-onderdrukkende functie door het activeren van PARP1 en DNA-herstel wordt vermoed. We vonden inderdaad dat lage nucleaire expressie geassocieerd was met een slechte prognose bij patiënten met stadium III MSS (microsatelliet stabiele) darmkanker die werden behandeld met op 5-FU gebaseerde adjuvante chemotherapie. Bovendien kan een hoge expressie van MACROD2 dienen als een predictieve biomarker in stadium III MSS-tumoren, met een uitgesproken voorkeur voor behandeling met adjuvante chemotherapie bij deze patiënten.

In het laatste hoofdstuk, Hoofdstuk 6, beschrijven we een ultieme poging om de identificatie van hoog-risico patiënten te verbeteren. In ons cohort zijn in de loop der jaren verschillende biomarkers bestudeerd, sommige met veelbelovende en sommige met teleurstellende resultaten. Voor deze studie wilden we alle markers tesamen analyseren, om erachter te komen welke (combinatie van) biomarkers de prognose het beste konden inschatten, en of eerdere minder belangrijke biomarkers interessanter werden. Hoewel er ook andere technieken

werden gebruikt op (subsets van) dit cohort, d.w.z. massaspectrometrie, next generation sequencing en morfologische analyse (MVD), besloten we voor deze studie om te streven naar een haalbare en breed en overal toepasbare techniek, zoals immunohistochemie. Daarom werden alle eerder onderzochte immunohistochemische biomarkers op tissue microarrays (TMA's) opgenomen, naast alle algemene klinische en pathologische parameters die beschikbaar waren in dit cohort. Deze werden opgenomen in onze Classification and Regression Tree (CART) -analyse, die aantoonde dat zowel lymfovasculaire invasies (LVI) als KCNQ1 de belangrijkste kenmerken waren voor het inschatten van de prognose bij stadium II en III colonkanker.

CHAPTER 9

Appendices

9.1 SUPPLEMENTARY DATA – METHODS & TABLES

Chapter 2 – MVD

The entire manuscript, including links to the supplemenatary data can be found online (directly at https://bmcgastroenterol.biomedcentral.com/articles/10.1186/s12876-019-1063-4#Sec15, or via pubmed using the PMID 31420015). These supplementary data include the script for MVD-analysis, and 2 supplementary tables including clinicopathological parameters (baseline) en correlation between MVD and TSP, HIF1a and VEGFa.

Chapter 3 - CDX2

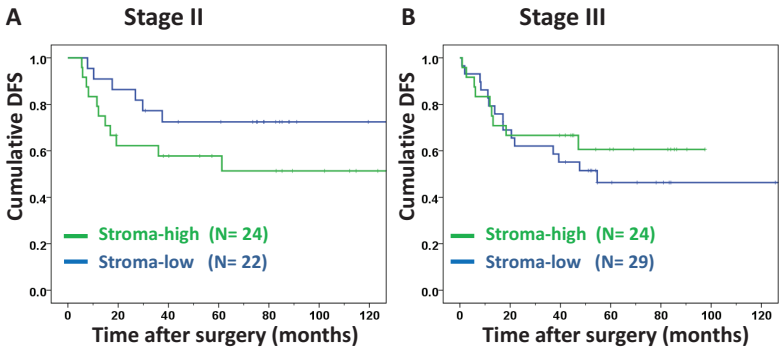
The manuscript and supplementary data of the manuscript concerning CDX2 can be found online (directly at https://doi.org/10.1016/j.ejca.2020.10.029, or via pubmed using PMID 33341450). These include Immunohistochemical straining protocol CDX2, materials & methods for proteomics and our statistical analysis.

Chapter 6 - CART

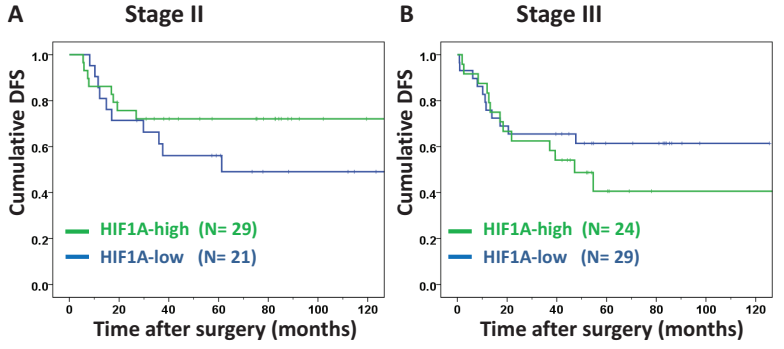
The manuscript, including links to the supplemenatary data can be found online (directly at https://bmccancer.biomedcentral.com/articles/10.1186/s12885-022-09473-9, or via pubmed using PMID 35395779). These date includes an overview of all biomarkers previously published on this cohort, the biomarker workflow and a script for RStudio (CART-analysis).

9.2 SUPPLEMENTARY DATA - FIGURES

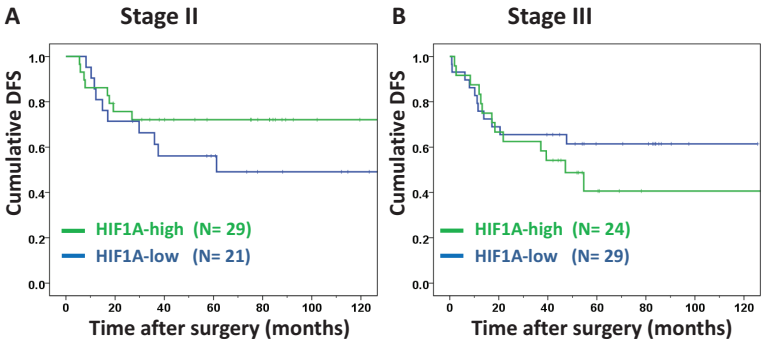
Chapter 2 – MVD



Supplementary Figure 1: Kaplan Meier curves for DFS of stage II (A) and stage III (B) colon cancer patients, stratified for high and low stromal percentage. Hazard ratio (HR), 95% confidence interval, and log-rank p-values are reported.

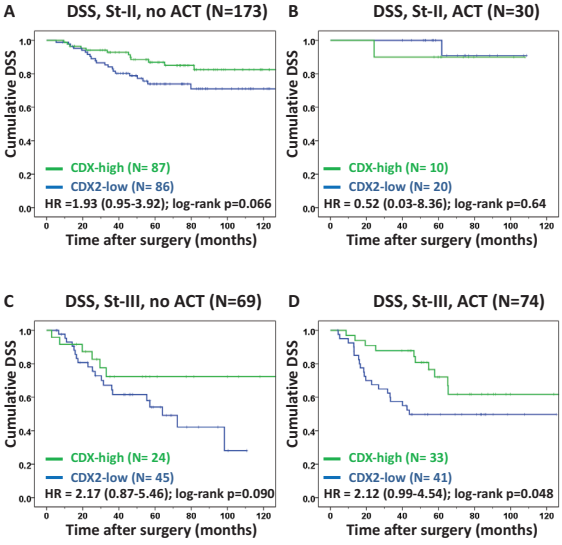


Supplementary Figure 2: Kaplan Meier curves for DFS of stage II (A) and stage III (B) colon cancer patients, stratified for high and low expression of HIF1A. Hazard ratio (HR), 95% confidence interval, and log-rank p-values are reported.

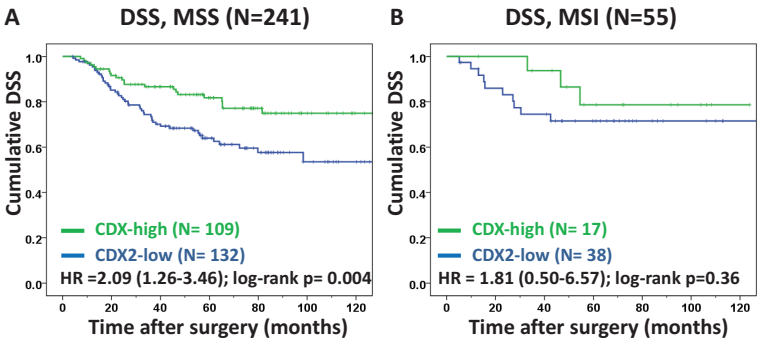


Supplementary Figure 3: Kaplan Meier curves for DFS of stage II (A) and stage III (B) colon cancer patients, stratified for high and low expression of VEGFA. Hazard ratio (HR), 95% confidence interval, and log-rank p-values are reported.

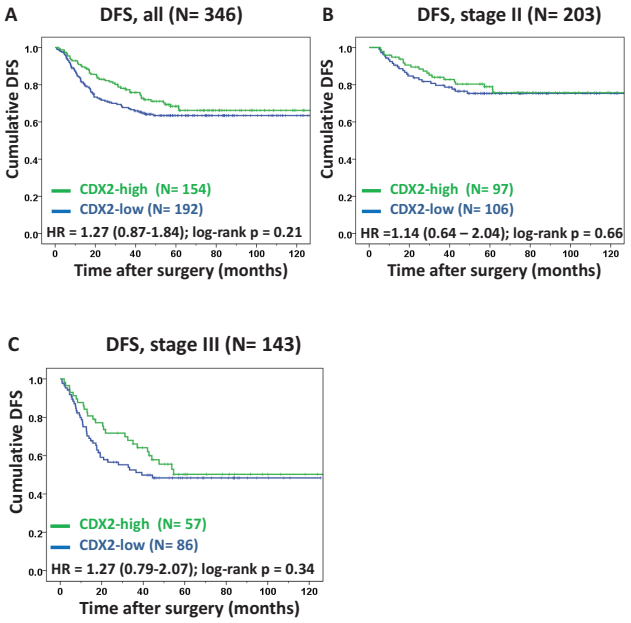
Chapter 3 - CDX2



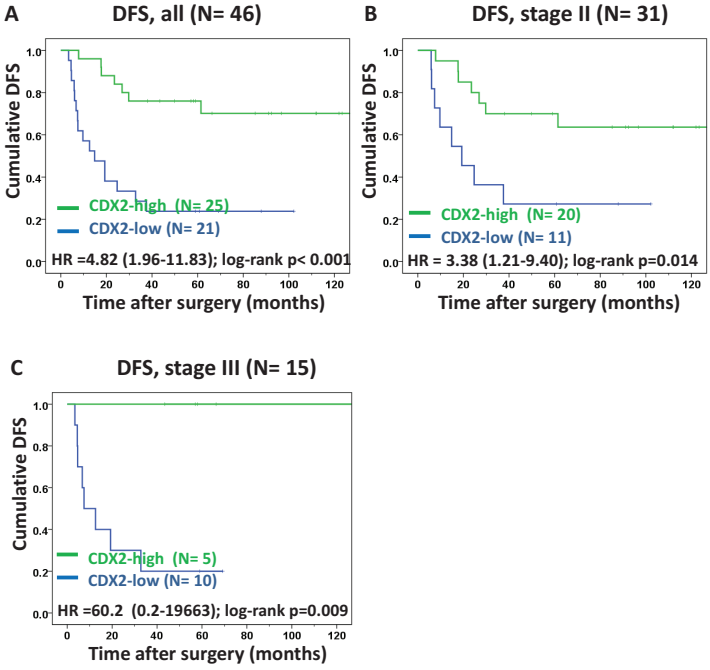
Supplementary figure 1: Association of CDX2 expression based on immunohistochemical detection with DSS of colon cancer patients. Stratification for CDX2-high and CDX2-low expression in stage II (A,B) and III (C,D) colon cancer patients treated without (A,C) and with (B,D) adjuvant chemotherapy (ACT). Cox regression HR (95% CI) and log-rank P-values are reported.



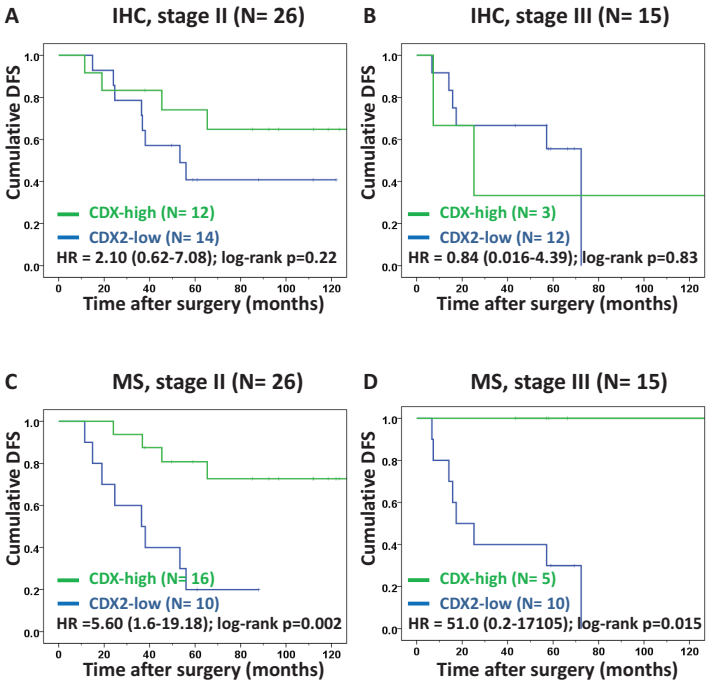
Supplementary figure 2: Association of CDX2 expression based on immunohistochemical detection with DSS of colon cancer patients. Stratification for CDX2-high and CDX2-low expression in MSS colon cancer patients (A); and in MSI colon cancer patients (B). Cox regression HR (95% CI) and log-rank P-values are reported.



Supplementary figure 3: Association of CDX2 expression based on immunohistochemical detection with DFS of colon cancer patients. Stratification for CDX2-high and CDX2-low expression in all stage II and III colon cancer patients (A); in stage II colon cancer patients (B); and in stage III colon cancer patients (C). Cox regression HR (95% CI) and log-rank P-values are reported.

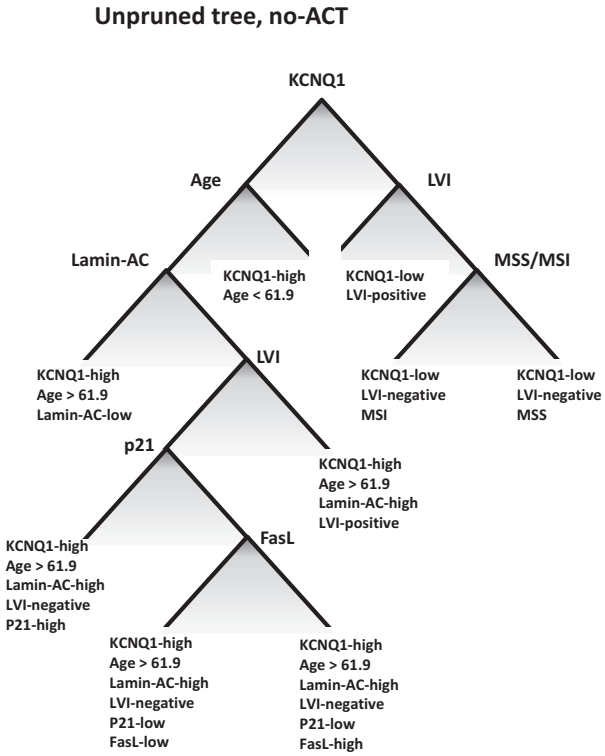


Supplementary figure 4: Association of CDX2 expression based on mass spectrometry with DFS of colon cancer patients. Stratification for CDX2-high and CDX2-low expression in all stage II and III colon cancer patients (A); in stage II colon cancer patients (B); and in stage III colon cancer patients (C). Cox regression HR (95% CI) and log-rank P-values are reported.

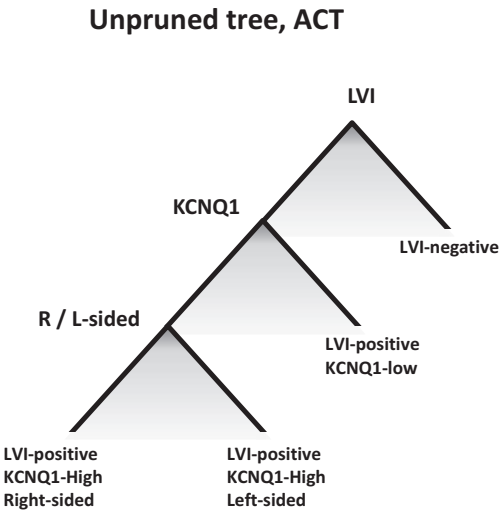


Supplementary figure 5: Association of DSS with CDX2 expression based on immunohistochemical (=IHC) detection (A, B) and mass spectrometry (=MS) (C, D) in the same subset of 41 patients, for both stage II (A, C) and stage III (B, D). Cox regression HR (95% CI) and log-rank P-values are reported.

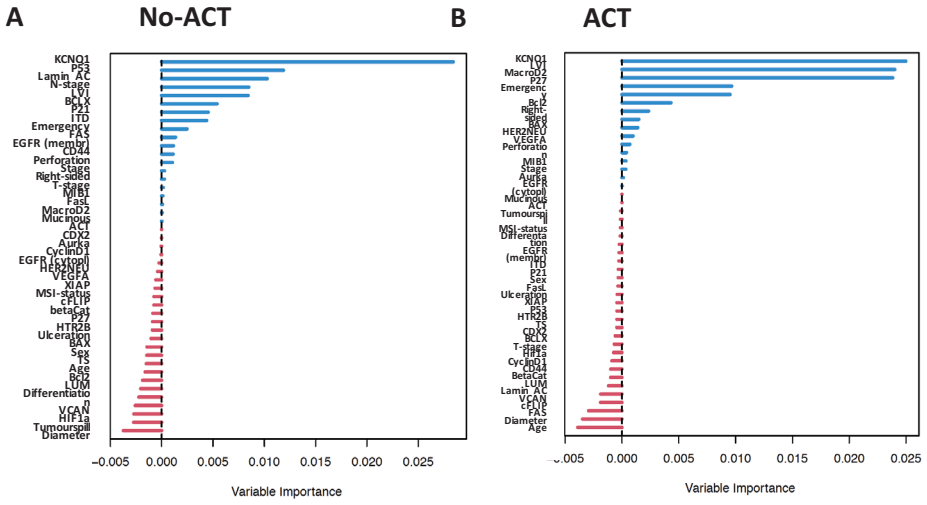
Chapter 6 - CART



Supplementary Figure 1: Unpruned classification tree by CART analysis for ACT-untreated patients



Supplementary Figure 2: Unpruned classification tree by CART analysis for ACT-treated patients.



Supplementary Figure 3: Variable importance graphs for both ACT-untreated and -treated patients. LVI = lymphovascular invasion / ITD = isolated tumour deposits / Emergency = emergency surgery / ACT = adjuvant chemotherapy.

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KCNQ1 and lymphovascular invasion are key features in a prognostic classifier for stage II and III colon cancer. *Sjoerd H. den Uil, Veerle M.H. Coupe, Herman Bril, Gerrit A. Meijer, Remond J.A. Fijneman, Hein B.A.C. Stockmann.* BMC Cancer 22, 372 (2022).

High expression of Secretory Leukocyte Protease Inhibitor (SLPI) in stage III micro-satellite stable colorectal cancer treated with adjuvant chemotherapy

is associated with reduced disease recurrence. *Sandrine Nugteren,* ***Sjoerd H. den Ui****l, Pien M. Delis-van Diemen, Ytje Simons-Oosterhuis, Dicky J. Lindenbergh-Kortleve, Daniëlle H. van Haaften, Hein B.A.C. Stockmann, Joyce Sanders, Gerrit A. Meijer, Remond J.A. Fijneman, Janneke N. Samsom.* Scientific reports. 2022 Jul 16;12(1):12174.

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Lieve **oma Gre,** ik ben ervan overtuigd dat als u 50 jaar later geboren was, u ook een dergelijk promotie-boekje had geschreven. Uw oprechte interesse in alles en iedereen is een voorbeeld voor mij en de familie. Lieve **Anne**, mijn jongste zusje. De grote afstand maakt je niet minder geïnteresseerd, betrokken of geliefd. Ik ben trots op de stoere keuzes die je maakt in je leven, en dat je ondanks de afleiding van het prachtige Canada stug aan je eigen onderzoek en carrière doorwerkt. En dan mijn ouders, lieve **Thea en Henk.** Wie had gedacht dat dat kleine blonde knaapje uit Koedijk geneeskunde zou gaan doen, en zich zou ontpoppen tot een academisch traumachirurg. En dan (uiteindelijk) nog promoveren ook. Dat had ik nooit kunnen doen zonder jullie onvoorwaardelijke steun. Niet alleen op persoonlijk vlak maar ook bij het faciliteren van een academische opleiding zijn jullie er altijd voor ons geweest, en zijn dat ook nu nog. En hoewel het 'medische wereldje' van buitenaf soms moeilijk te begrijpen is, zijn jullie altijd betrokken, geïnteresseerd en meelevend geweest. Enne, het is toch cool om te kunnen zeggen dat jullie als "VU-stel' ook alle 3 de kids aan de Vrije Universiteit

hebben laten studeren. Woorden schieten te kort om mijn dankbaarheid te verwoorden. Ik hou van jullie, geniet van jullie pensioen!

En dan mijn zoon, lieve Luuk. Zonder dat jij het wist was je voor mij het laatste zetje in de rug om dit proefschrift af te ronden. Ik verheug me om je op te zien groeien, op de tijd die we samen gaan doorbrengen en ik ben benieuwd welk carrière-pad je ooit zult gaan volgen. En mocht je ooit willen gaan promoveren, nee laat maar, daar hebben we het tegen die tijd wel over.

En dan tot slot, mijn lieve **Tessa.** Mijn steun, mijn liefde, mijn partner en de moeder van mijn zoon, niet noodzakelijkerwijs in die volgorde. Wat hebben wij een fijn gezin en een mooi leven. Je hebt me altijd gesteund, zowel qua werk als daarbuiten. Ik kan me niet voorstellen hoe ik dit boekje zonder jou voor elkaar had gekregen. Ik bewonder hoe attent en sociaal je bent, en ben trots op hoe je jouw carrière vorm weet te geven en de ruimte die we elkaar hiervoor geven. Ik ben dankbaar voor wie je (voor mij en Luuk) bent, en ik kijk uit naar alles wat het leven nog voor ons in petto heeft. Ik hou van je!

CURRICULUM VITAE

Sjoerd Hendrik den Uil was born on January 20th 1987 in Alkmaar, the Netherlands, as the first child of two academically trained high-school teachers (German and Mathematics). After graduating Gymnasium from *C.S.G. Jan Arentsz* he started medical school in 2005 at the Vrije Universiteit in Amsterdam. He participated in pharmacotherapy teaching, examination and research. Towards the end he chose an elective course in anatomy, and general surgery as his final internship. Graduating in 2011, he started his career as a medical doctor in the surgical department of the *Kennemer Gasthuis,* where he applied for a PhD-position, that continued to one fulltime year of research in the *Cancer Centre Amsterdam* in 2014, sponsored by the local surgical foundation (Stichting Chirurg en Onderzoek Kennemerland) and the department of pathology of the *VU university medical centre*. In 2015 he started his surgical residency, that led him through six years of training in, by that time, the *Spaarne Gasthuis* and the *VU university medical centre.* In 2021 he registered as a trauma surgeon and surgeon in children, and he started his fellowship in trauma surgery at *location AMC* of the *Amsterdam University Medical Centre*, where he was able to obtain a position in the surgical trauma staff by January 2022.