A cohort study of sex differences and prognostic biomarkers in colorectal cancer

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2013

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

By due permission of the Faculty of Medicine, Lund University, Sweden

To be defended at the Main lecture hall, Department of Pathology
Sölvegatan 25, Skåne University Hospital, Lund
Friday 22nd of November 2013 at 09:15 am

Faculty opponent

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Helsinki, Finland

Organization	Document name
LUND UNIVERSITY	DOCTORAL DISSERTATION
	Date of issue
Author(s) Sakarias Wangefjord	Sponsoring organization

Title and subtitle: A cohort study of sex differences and prognostic biomarkers in colorectal cancer

Abstract

Colorectal cancer (CRC) is one of the most common forms of cancer worldwide, with an annual incidence of more than 1 million cases. Despite advancements in the management of CRC, mortality remains high. Accumulating evidence indicates that CRC is a heterogeneous disease, which affects outcome beyond what can be predicted by disease stage and other conventional prognostic factors. Thus, there is a great need to identify new biomarkers to enable a more accurate prognostication, and to help select patients for adjuvant treatment.

The aim of this thesis was to investigate the associations of a series of putative biomarkers with survival, treatment response and clinicopathological factors in a large cohort of incident CRC, with special attention to sex differences.

The study group consisted of tumours from 557 incident cases of CRC in the Malmö Diet and Cancer Study (MDCS). The tumours, assembled in tissue microarrays, were evaluated for expression of cyclin D1, mismatch repair proteins, beta-catenin and epidermal growth factor receptor (EGFR) by immunohistochemistry, and further, EGFR gene copy number (GCN) alterations by brightfield double-in situ hybridization. In addition, KRAS and BRAF mutational status was assessed by pyrosequencing. Associations with clinicopathological and investigative factors were explored by Chi Square and Spearman's correlation tests, and Kaplan-Meier analysis and Cox proportional hazards modelling were applied for survival analysis.

We hereby found that nuclear cyclin D1 expression was associated with female sex and a favorable prognosis, although not independently, in male, but not in female, CRC. Microsatellite instability (MSI) correlated to distinctive clinicopathological features, cyclin D1 expression, and independently predicted a good prognosis in stage III-IV CRC. Moreover, beta-catenin overexpression correlated independently with a prolonged survival from stage III-IV CRC, and was associated with microsatellite stable (MSS) tumours. We also observed that KRAS codon 13 mutation predicted a poor prognosis in female CRC, but not independently of established prognostic factors. KRAS and BRAF mutations were mutually exclusive, and correlated with MSS and MSI, respectively. BRAF mutation was independently associated with a reduced survival in male patients with MSS CRC. Furthermore, both EGFR protein expression and GCN alterations were associated with a reduced survival in stage III-IV CRC, the latter, however, not independently of established prognostic factors. EGFR protein expression correlated significantly with EGFR GCN alterations, although a substantial proportion of EGFR expressing tumours displayed a normal GCN, and vice versa. Finally, EGFR alterations were significantly associated with a reduced survival in curatively treated patients with stage III-IV disease receiving adjuvant oxaliplatin.

In conclusion, the results from this thesis demonstrate several relevant associations of the investigative biomarkers with prognosis and treatment response in CRC. Moreover, substantial sex differences in the clinicopathological correlates and prognostic significance of some of the biomarkers were observed.

Key words: colorectal cancer, prognosis, treatment prediction, biomarkers, sex differences, cyclin D1, microsatellite instability, beta-catenin, KRAS mutation, BRAF mutation, EGFR expression, EGFR gene copy number alterations

Classification system and/or index terms (if any)

Supplementary bibliographical information		Language: English
ISSN and key title 1652-8220		ISBN 978-91-87449-92-5
Recipient's notes	Number of pages	Price
	Security classification	

Signature _____ Date _____

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The research presented in this thesis was supported by:

The Knut and Alice Wallenberg Foundation, the Swedish Cancer Society, the Gunnar Nilsson Cancer Foundation, the Crafoord Foundation, the Swedish Government Grant for Clinical Research, Lund University Faculty of Medicine and Skåne University Hospital Research Grants.

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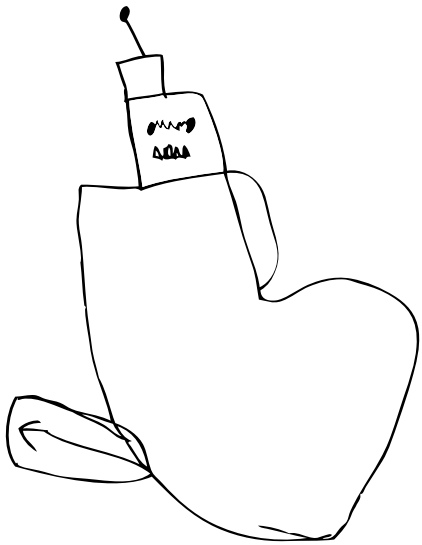
Lund University, Faculty of Medicine Doctoral Disssertation Series 2013:119
ISBN 978-91-87449-92-5
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2013

To my family



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Cyclin D1 expression in colorectal cancer is a favorable prognostic factor in men but not in women in a prospective, population-based cohort study.
Biol Sex Differ. 2011; 2:10

II.

Wangefjord S, Brändstedt J, Lindquist KE, Nodin B, Jirström K, Eberhard J
Associations of beta-catenin alterations and MSI screening status with expression of key cell cycle regulating proteins and survival from colorectal cancer.
Diagn Pathol. 2013; 8:10

III.

Wangefjord S, Sundström M, Zendehrokh N, Lindquist KE, Nodin B, Jirström K, Eberhard J
Sex differences in the prognostic significance of KRAS codons 12 and 13, and BRAF mutations in colorectal cancer: a cohort study.
Biol Sex Differ. 2013; 4:17

IV.

Wangefjord S, Elmberger G, Larsson A, Sundström M, Nodin B, Eberhard J, Jirström K
Impact of epidermal growth factor receptor protein expression and gene copy alterations on survival from colorectal cancer: a cohort study.
Submitted

Related papers not included in the thesis

Larsson A, Johansson ME, Wangefjord S, Gaber A, Nodin B, Kucharzewska P, Welinder C, Belting M, Eberhard J, Johnsson A, Uhlén M, Jirström K
Overexpression of podocalyxin-like protein is an independent factor of poor prognosis in colorectal cancer.
Br J Cancer. 2011; 105(5):666-72.

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A cohort study of the prognostic and treatment predictive value of SATB2 expression in colorectal cancer.
Br J Cancer. 2012; 106(5):931-8

Nodin B, Johannesson H, Wangefjord S, O'Connor DP, Lindquist KE, Uhlén M, Jirström K, Eberhard J
Molecular correlates and prognostic significance of SATB1 expression in colorectal cancer.
Diagn Pathol. 2012; 7:115

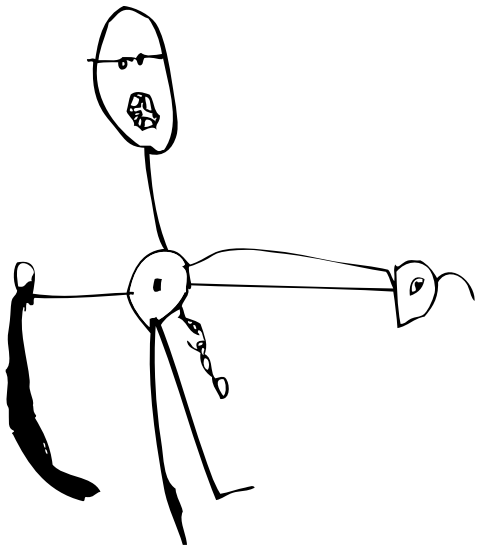
Brändstedt J, Wangefjord S, Nodin B, Gaber A, Manjer J, Jirström K
Gender, anthropometric factors and risk of colorectal cancer with particular reference to tumour location and TNM stage: a cohort study.
Biol Sex Differ. 2012; 16:3

Abbreviations

5-FU	Fluorouracil
ACF	Aberrant crypt foci
AJCC	American Joint Committee on Cancer
APC	Adenomatous polyposis coli
APR	Abdominoperineal resection
BDISH	Brightfield double-in situ hybridization
BRAF	V-raf murine sarcoma viral oncogene homolog B
cCR	Complete clinical response
CDK	Cyclin dependent kinase
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CEA	Carcinoembryonic antigen
CEN7	Chromosome 7 centromere
CI	Confidence interval
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CK1	Casein kinase 1
CME	Complete mesocolic excision
CRC	Colorectal cancer
CRT	Chemoradiotherapy
CSS	Cancer specific survival
CVL	Central vascular ligation
CT	Computed tomography
DCC	Deleted in colorectal cancer
DNA	Deoxyribonucleic acid

EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERβ	Estrogen receptor beta
FAP	Familial adenomatous polyposis
FLOX	Flourouracil + leucovorin + oxaliplatin
FLV	Flourouracil + leucovorin
FOBT	Fecal occult blood test
GCN	Gene copy number
GDP	Guanosine diphosphate
GSK3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
Gy	Gray
HIER	Heat induced epitope retrieval method
HNPCC	Hereditary nonpolyposis colorectal cancer
HR	Hazard ratio
HRT	Hormone replacement therapy
IBD	Inflammatory bowel disease
IHC	Immunohistochemistry
ISH	In situ hybridization
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LAR	Low anterior resection
LOH	Loss of heterozygosity
MAPK	Mitogen activated protein kinase
mCRC	Metastatic colorectal cancer
MDCS	Malmö Diet and Cancer Study
MGMT	O-6-methylguanine-DNA methyltransferase
MLH1	mutL homolog 1
MMR	Mismatch repair
MRI	Magnetic resonance imaging

MSH2	mutS protein homolog 2
MSH6	mutS homolog 6
MSI	Microsatellite instability / unstable
MSS	Microsatellite stable
NSAID	Non-steroidal anti-inflammatory drugs
OC	Oral contraceptives
PCR	Polymerase chain reaction
PET	Positron emission tomography
PMS2	Postmeiotic segregation increased 2
RNA	Ribonucleic acid
rRb	Retinoblastoma protein
RT	Radiation therapy
SSA	Sessile serrated adenoma
TEM	Transanal endoscopic microsurgery
TGF-α	Transforming growth factor alpha
TMA	Tissue microarray
TME	Total mesorectal excision
TNM	Tumor-node-metastasis classification of malignant tumours
UICC	International Union for Cancer Control
VEGF	Vascular endothelial growth factor
WHI	Women's Health Initiative
XELOX	Capecitabine + oxaliplatin



Background

Colorectal cancer

Epidemiology

Colorectal cancer (CRC) is the third most common cancer in men and the second most common cancer in women globally, with an annual incidence of approximately 1.2 million cases and more than 600000 related deaths each year [1]. Incidence rates vary largely by geographical location, with the highest rates reported in more developed regions, i.e. North America, Western Europe, Australia and New Zeeland, whereas the lowest rates are seen in Africa (except Southern Africa) and South-Central Asia [1]. The incidence of CRC is substantially higher in men than in women, with a 1.4:1 male to female rate globally [1]. Swedish incidence rates for colon cancer are similar between the sexes, but for rectal cancer, the incidence is approximately 50% higher in men than in women [2,3].

Increasing incidence rates are observed in newly developed or economically transitioning countries, most likely reflecting a "Westernization" of the lifestyle with altered dietary habits, increased prevalence of obesity and decreased physical activity [4]. During the last decades, CRC incidence rates, adjusted for population growth and increased life expectancy, have been fairly stable, with only a modest annual increase in Sweden [5,6], and other developed countries [4,7]. Interestingly, CRC incidence rates are declining in North America [8], largely attributed to the implementation of screening [9,10].

In the last decades, CRC age-standardized mortality rates have steadily decreased, and survival has consistently increased, in Sweden [6] and the rest of the developed world [4,11,12]. These trends are likely due to the to the improvements made in CRC detection and treatment during this period [13-15]. Mortality rates are generally lower in women than in men [1].

The relative 5-year survival rates for colon cancer in Sweden 2011 were 64.1% for men and 66.8% for women. For rectal cancer, the corresponding relative 5-year survival rates were 62.9% for men and 64.2% for women [16].

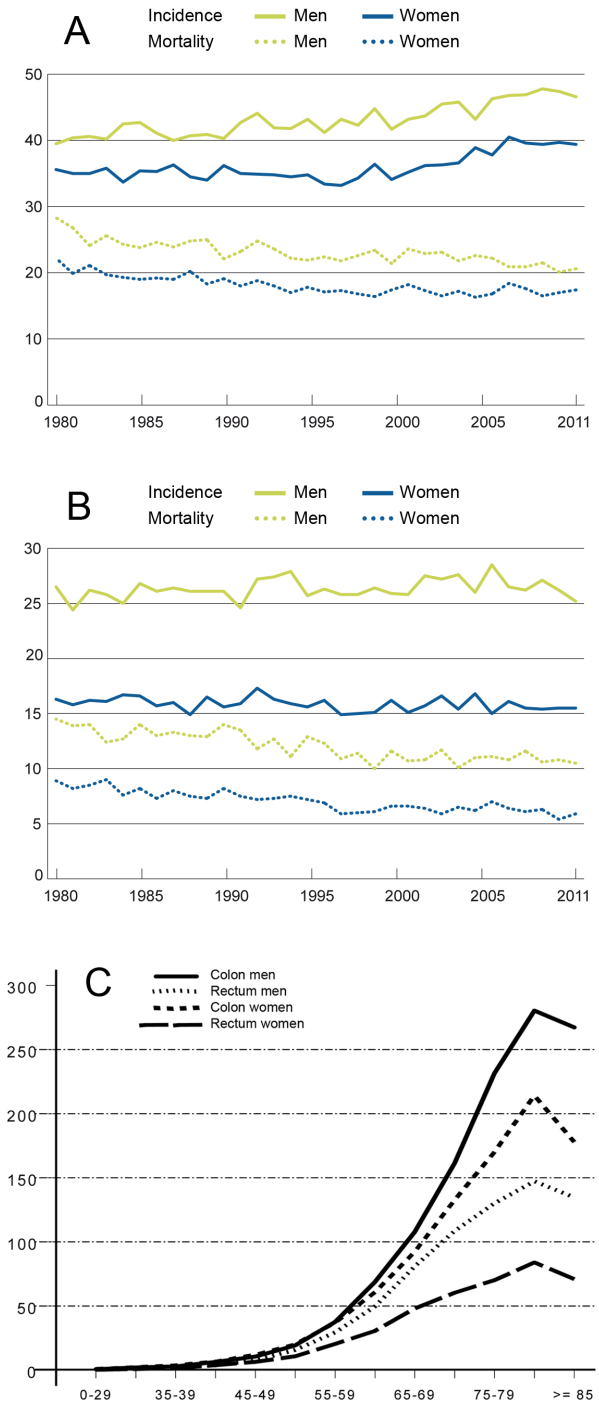


Figure 1. The age standardized incidence and mortality for colon cancer (A), rectal cancer (B), and age specific incidence of CRC (C) in Sweden [16,17].

Etiology and risk factors

CRC most commonly occurs sporadically, and, as with the majority of other malignancies, the etiology is multifactorial [18]. CRC risk increases with age and 75% of cases occur after the age of 65 [17]. The rapid increase in CRC incidence in populations previously considered to be at low risk, the substantial geographical difference in incidence, and the incidence changes observed in migrant studies suggest environmental and lifestyle factors as etiological agents [19]. Diet is likely the most important exogenous factor and the evidence that consumption of red meat, processed meat and excessive amounts of alcohol causes CRC is convincing [20]. Further, food containing dietary fibers seems to protect from CRC [20].

Obesity, and in particular abdominal fatness, likely increases the risk of CRC, whereas physical activity decreases the risk [20]. Interestingly, the increased risk for CRC associated with obesity is more pronounced in men and premenopausal women [21]. Accumulating evidence further supports that tobacco smoking is a risk factor for CRC [19,22,23]. Other environmental factors include cholecystectomy, that has been proposed to increase the risk for CRC by means of altered bile secretion, but results are conflicting [24-26]. The association between inflammatory bowel disease (IBD) and CRC is well established and forms the basis for widely adopted endoscopic surveillance recommendations [27,28]. However, recent findings suggest that the increase in risk for CRC due to IBD might not be as substantial as previously considered [29,30].

Long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) has been proven to significantly reduce the risk for CRC [31], and prophylactic NSAID treatment in high-risk groups, i.e. patients with inherited CRC predisposition, is feasible [32]. Serious side effects, such as bleeding ulcer, may however outweigh the benefits for the general public [33].

Results from the Women's Health Initiative (WHI) large randomized controlled trials of hormone therapy have demonstrated that postmenopausal hormone replacement therapy (HRT), with estrogen plus progestin, significantly reduces the incidence of colon cancer [34]. Of note, women who took estrogen plus progestin were diagnosed at a more advanced stage than those who took placebo [34]. Interestingly, therapy with estrogen alone was not significantly related to CRC incidence [35]. Moreover, the risk for breast cancer, coronary heart disease, stroke, and venous thromboembolic disease were increased in women taking estrogen plus progestin [34], hence limiting the potential use of HRT as chemoprevention for CRC.

Hereditary CRC

Approximately 20-30% of CRC cases have a familiar basis, that is two or more first- or second degree relatives (or both) also having been diagnosed with CRC, whereas highly penetrant, inherited syndromes such as hereditary nonpolyposis colon cancer (HNPCC) and familial adenomatous polyposis (FAP) account for less than 5% of cases [18,36].

HNPCC, also referred to as the Lynch syndrome, is the most common form of hereditary CRC [36]. HNPCC is caused by a germline mutation in one of the genes associated with the DNA mismatch repair (MMR) system, predominantly MLH1, MSH2, MSH6 or PMS2, leading to microsatellite instability (MSI) [36]. Inheritance is autosomal dominant, and individuals carrying mutations in the MMR genes have a 50–80% lifetime risk of developing CRC [18]. Carcinogenesis is markedly accelerated in HNPCC, and CRC commonly has an early age of onset, with a mean age of 45 years [36]. HNPCC tumours are preferably located in the proximal colon, and display specific histopathologic characteristics, i.e. poor differentiation, mucinous or signet ring cell histology, and marked lymphocytic infiltration [36]. Multiple synchronous and metachronous CRCs are common [36], and there is an excess of extracolonic malignancies, predominantly endometrial cancer [36]. Individuals at risk can be identified by assessment of personal and family cancer history and by molecular testing of CRC tumor specimens for MSI [18]. The diagnosis is confirmed by germline MMR gene mutation analysis [18].

FAP is characterized by hundreds to thousands of adenomatous polyps that develop at an early age. The condition is caused by a germline mutation in the adenomatous polyposis coli (APC) gene and is most often inherited in an autosomal dominant manner [18]. However, up to 30% of cases emerge as de novo mutations, and, consequently, are not associated with a family history [37]. Without prophylactic surgery, the risk of developing CRC is nearly 100% by the age of 40 [37]. Extra-colonic manifestations include duodenal adenomas, and, less commonly, desmoids and osteomas [37]. Patients with a verified APC mutation, or a familiar history of FAP, should be surveilled with endoscopy and recommended prophylactic surgery once the polyp burden is too extensive [37]. Total proctocolectomy with ileoanal pouch anastomosis is the preferred approach rather than less extensive surgery, such as total colectomy with ileorectal anastomosis, as the remaining mucosa is at substantial risk for malignant transformation [37].

Carcinogenesis

For decades, the paradigm of CRC carcinogenesis has been the multistep genetic model proposed by Fearon and Vogelstein in 1990. In brief, a series of genetic events were postulated to be required for the development of CRC. These events

include mutational activation of several oncogenes and inactivation of tumour suppressor genes, leading to a stepwise accumulation of genetic alterations, ultimately resulting in malignant transformation [38]. Morphologically, these events are reflected in the sequential transformation of normal mucosal epithelium through adenoma to carcinoma, widely described as the "adenoma-carcinoma sequence" [39]. Although this model is still definitely valid, it is not telling the whole truth. Most importantly, it has become clear that the genetic alterations required for colorectal carcinogenesis may develop through several different pathways, and, therefore, the transformation from normal epithelium to cancer obviously is not as sequential and uniform as previously suggested.

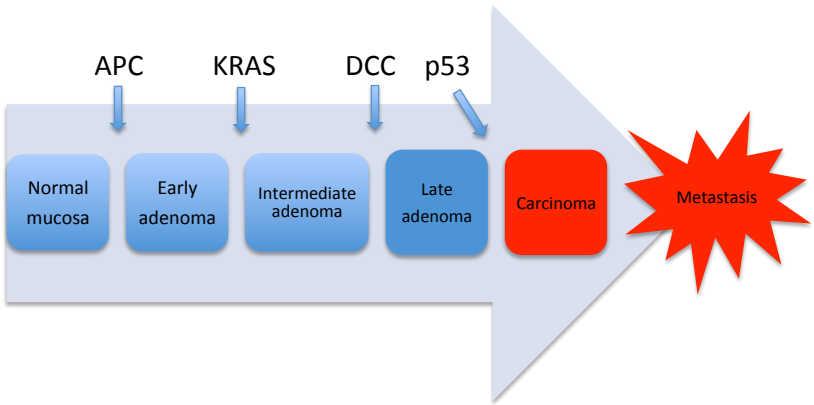


Figure 2. The adenoma carcinoma sequence, as proposed by Fearon and Vogelstein. Tumourigenesis proceeds through a series of genetic alterations involving oncogenes and tumour suppressor genes.

Early premalignant changes have been observed in the mucosal crypts. These so called aberrant crypt foci (ACF) harbor premalignant genetic alterations and are considered to be precursor lesions to adenomas and carcinomas [40]. Likely, ACF can also transform directly to cancer, bypassing the adenoma stage [40]. However, most CRCs evolve in a preexisting adenoma [41], and this malignant transformation generally takes decades. However, time to progression varies depending on the type of adenoma, with large size, multiple adenomas, villous histology and high-grade dysplasia being high-risk features [42]. Further, sessile serrated adenomas (SSA), comprising a subgroup with distinct molecular and pathological characteristics, are thought to progress to cancer via a different pathway [43].

An underlying genetic instability is considered to be a prerequisite for the significant accumulation of genetic and epigenetic alterations seen in CRC [44]. At present, three different major pathways of genetic instability have been recognized; i.e. the chromosomal instability (CIN), the microsatellite instability (MSI) and the CpG Island Methylator Phenotype (CIMP) pathways [45]. Depending on the carcinogenetic pathway, CRCs acquire distinct different molecular, pathological and clinical characteristics. However, these pathways are not always mutually exclusive, and a tumour can exhibit features from different pathways [46].

The chromosomal instability (CIN) pathway

Approximately 70-85% of CRCs develop through the CIN pathway [47,48]. CIN is characterized by an accelerated rate of gains or losses of whole or large portions of chromosomes that result in intercellular karyotypic variability [46]. The consequence of CIN is aneuploidy, subchromosomal genomic amplifications, and a high frequency of loss of heterozygosity (LOH) [46]. CIN is further associated with a characteristic set of mutations, most importantly mutation in APC and/or loss of chromosome 5q, harbouring the APC gene, mutation of the v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) oncogene, loss of chromosome 18q and deletion of chromosome 17p, harboring the tumour suppressor gene TP53 [48].

The crucial tumour suppressor gene APC is mutated in up to 80% of sporadic CRCs and adenomas, indicating that this mutation represents an early event in carcinogenesis [46,48]. By binding to beta-catenin, APC suppresses activation of the Wnt-signaling pathway that regulates cellular growth, apoptosis and differentiation [48]. Mutations in APC truncate the APC protein and interrupt its binding to beta-catenin, thus causing unsuppressed downstream signaling [46].

KRAS is another important gene within the CIN pathway. In brief, mutated KRAS is locked in a constitutively active form, and downstream regulation of the Ras/Raf/MEK/MAPK pathway is lost [49]. Activating KRAS mutations are found in approximately 40% of CRCs [49].

Finally, inactivation of TP53 occurs either through loss of heterozygosity or mutation and is considered a late event in colorectal carcinogenesis [46]. The p53 protein normally acts to increase the expression of cell-cycle inhibiting genes upon DNA damage, in order provide sufficient time for DNA repair. Furthermore, when the genetic damage is irreparable, p53 induces pro-apoptotic genes, thus terminating the genetic insult through programmed cell death [46].

Tumours of the CIN phenotype develop though the traditional adenoma-carcinoma sequence and exhibit clinical characteristics such as distal location and high differentiation grade, and have an intermediate prognosis [47,50,51].

The microsatellite instability (MSI) pathway

Microsatellites are short repeating sequences of DNA, spread out over the whole genome and are prone to errors during replication due to their repetitive manner [47]. Mismatches of nucleotides occur when the DNA-polymerase inserts bases wrongly in the newly synthesized DNA. The DNA mismatch repair system (MMR) acts as a ”spell checker”, normally recognizing and repairing these mismatches instantly [47]. Instability of microsatellites is a reflection of the inability of the MMR system to correct these errors and is recognized by frameshift mutations in the microsatellite repeats [52].

The pure form of MSI is caused by a germline mutation in one of the MMR genes, as seen in HNPCC. However the majority of MSI CRCs occur sporadically due to DNA methylation of the MLH1 promoter and the consequent transcriptional silencing of MLH1 expression. In other words, MSI is acquired by the CIMP pathway in sporadic CRC [47,48,53]. MSI tumours, whether sporadic or inherited, share similar biology [48], however the precursor lesion is generally a traditional adenoma in HNPCC and a CIMP-associated SSA in sporadic MSI [48]. Approximately 15% of sporadic CRCs display MSI [52].

Detection of MSI can be done either indirectly by demonstrating the absence of expression of MMR proteins with immunohistochemistry (IHC) or more directly by polymerase chain reaction (PCR) based amplification of specific microsatellite repeats [54]. When evaluating MSI status by IHC, a tumour is generally classified as being either microsatellite unstable (MSI) or microsatellite stable (MSS), depending on the expression of MMR proteins MLH1, PMS2, MSH2 and MSH6 [54]. In PCR based MSI analysis, a panel of five specific microsatellite loci (BAT25, BAT26, D5S346, D2S123, and D17S250) are generally used and tumours classified as either MSI-high, when instability is observed in at least two markers, MSI-low when unstable in one marker, or MSS when there is no apparent instability [54]. However, MSI-low CRCs do not appear to differ clinically or pathologically from MSS CRCs, and generally MSI-low is categorized as MSS [54]. The validity of the two methods has been debated, but both are well recognized and have proven concordant [54].

MSI tumours have distinct clinical and pathological characteristics; tumours are predominantly located in the proximal colon and typically display poor differentiation, mucinous or signet ring cell histology, and marked lymphocytic infiltration [53]. In sporadic CRC, MSI is further associated with female sex and old age [52]. Despite the adverse pathological features, MSI is generally associated with a good prognosis [55].

MSI tumours tend to be diploid, with less LOH and few mutations in KRAS and p53 [52]. BRAF V600E mutation is commonly seen in sporadic MSI CRC, but very seldom in HNPCC [52].

In vitro studies indicate resistance of MSI tumors to various chemotherapeutic agents, such as fluorouracil (5-FU) and cisplatin [56], but clinical data on the use of MSI as a chemotherapy predictive marker are conflicting [55,57].

The CpG Island Methylator Phenotype (CIMP) pathway

Epigenetic alterations refer to changes in gene expression or function that are mediated by mechanisms that do not affect the DNA sequence [47]. These changes are usually caused by DNA methylation or histone modifications [47].

DNA methylation commonly occurs in short sequences rich in the CpG dinucleotide, so called CpG islands, which can be found in the promoter regions in about half of all human genes [58]. Methylation of cytosines within these regions causes loss of gene expression, functionally equivalent to inactivating mutation [58]. Aberrant DNA methylation increases with age and can also be induced by environmental factors, such as smoking [52].

Several important tumour suppressor genes, such as APC, CDKN2A, MGMT and MLH1, can be silenced by DNA methylation in CRC [52,59]. A subset of CRCs exhibits widespread CpG hypermethylation, referred to as the CpG island methylator phenotype (CIMP) [58].

Using PCR-based methods, the presence of methylation in a panel of CpG markers can be assessed [48]. Most often, tumours are categorized as either CIMP-high, CIMP-low or CIMP-negative, depending on the extent of methylation [60]. CIMP-low has been associated with KRAS mutation and male sex [61], however it is controversial whether CIMP-low represents a distinct entity or not [59].

Tumours with CIMP constitute a distinct subgroup, and are associated with proximal location, female sex, old age, mucinous and poor differentiation, MSI, BRAF mutation and inversely associated with TP53 mutation [52,59]. Up to 20% of sporadic CRCs are CIMP-high [52].

CpG methylation is an early event in CRC carcinogenesis [47,61], and the precursor lesion is generally a SSA [52].

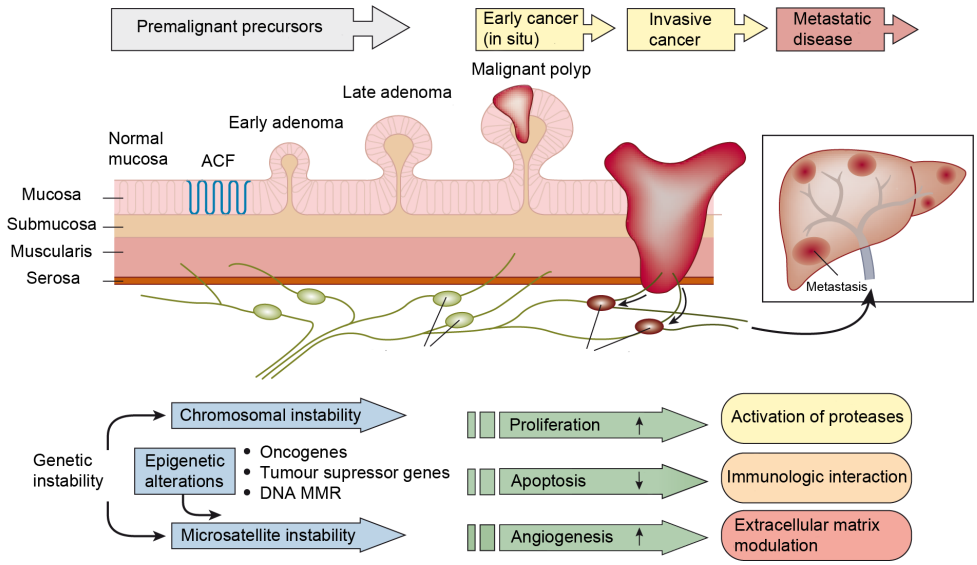


Figure 3. Schematic model of CRC carcinogenesis. The transformation from normal mucosa to metastatic cancer involves multiple molecular alterations in complex interaction. Used with permission by Tidsskrift for Den norske legeforening [62].

In summary, CRC evolves through at least three distinct pathways that can be defined by certain molecular features, i.e. CIN, MSI and CIMP. These entities are not mutually exclusive, but CIN seems to exclude CIMP [51]. A classification of CRC based on these molecular characteristics has been suggested [60]:

1. CIMP high/MSI high (12% of CRC); originates in serrated adenomas and is characterized by BRAF mutation and MLH1 methylation.
2. CIMP high/MSI low or MSS (8%); originates in serrated adenomas and is characterized by BRAF mutation and methylation of multiple genes.
3. CIMP low/MSI low or MSS (20%); originates in tubular, tubulovillous, or serrated adenomas and is characterized by CIN, KRAS mutation, and MGMT methylation.
4. CIMP negative/MSS (57%); originates in traditional adenoma and is characterized by CIN.
5. HNPCC; CIMP negative/MSI high (3%); negative for BRAF mutations.

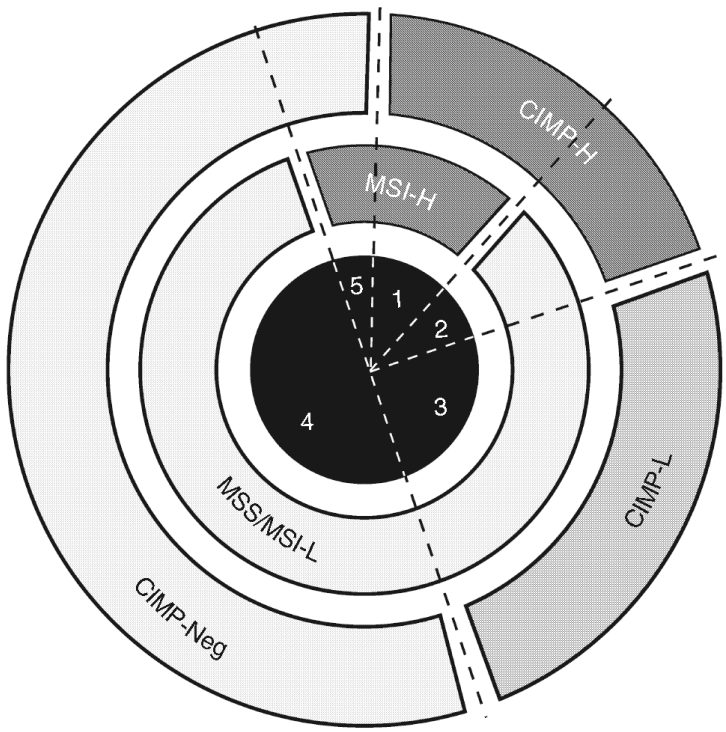


Figure 4. Derivation of molecular CRC groups 1-5 based on CIMP status (H=high, L= low, Neg=negative) and MSI status (H=high, L=low, S=stable). Used with permission by John Wiley & Sons Ltd [60].

Sex differences in carcinogenesis, risk and survival

To begin with, several sex-related differences can be observed in CRC epidemiology. As previously noted, the CRC incidence and mortality rates are significantly higher in men than in women [1]. The discrepancies in incidence and mortality are particularly striking between premenopausal women and age-matched men [63], indicating a hormonal influence. Further, differences in dietary and lifestyle factors likely contribute [64]. Among known risk factors for CRC, women appear to ingest more dietary fibre, seem to benefit more from physical activity, and consume less alcohol [64].

CRC mortality rates are declining in most developed countries, particularly in women [4,65]. A possible explanation for the comparatively improved survival in women might be earlier and more widespread favorable dietary and lifestyle habits [65], but may also be due to the use of postmenopausal HRT or oral contraceptives (OC) [65]. Other factors potentially accounting for sex differences in survival are differences in screening participation and stage at diagnosis [66].

Further, women more often have proximal tumours [63], and are more likely to respond to adjuvant treatment with 5-FU [63]. At the molecular level, MSI and CIMP-high tumours are more frequent in women [67].

The metabolic syndrome has been reported as an independent risk factor for CRC in men but not in women [68], and obesity seems to increase the risk for CRC, in particular in men [21]. Obese men further appear to present with a more advanced tumour stage at diagnosis [69].

Clinical management

The successful management of CRC is truly a multidisciplinary task, and depends upon the cooperation between pathologists, radiologists, oncologists and surgeons to ensure a detailed diagnosis, optimal surgery and adequate adjuvant treatment. Surgery is the cornerstone for curative treatment and has changed considerably over the last decades. Most importantly, the implementation of the total mesorectal excision (TME) technique has improved the outcome significantly for patients with rectal cancer [70]. Advances in neoadjuvant and adjuvant chemo-radiotherapy have increased survival and reduced recurrences, and the addition of targeted therapies has prolonged life in metastatic disease to a considerable extent [71-73]. Further, imaging modalities such as magnetic resonance imaging (MRI) and positron emission tomography (PET) have contributed to better staging. Of note, no prognostic molecular biomarkers have yet found a place in clinical protocols.

Prevention

Primary prevention of CRC means the identification and removal of etiological risk factors [17]. In specific, there is convincing evidence that obesity, physical inactivity, smoking, a diet low in fiber and high in red meat and excessive alcohol consumption increases the risk for CRC [20,74]. Thus, counteracting lifestyle modifications likely have a significant impact on CRC development and should be recommended. Moreover, both the use of NSAIDs and postmenopausal HRT has proven to increase the risk of CRC, however side effects limit the clinical use as chemopreventive agents [33,34].

Secondary prevention of CRC aims to identify cases with subclinical disease by screening in a healthy population, i.e. to detect and remove premalignant adenomas before the development of CRC [17,75].

Screening has proven to significantly reduce the relative mortality from CRC [76,77] and several methods can be used. Indirect methods include fecal occult blood tests (FOBT) and stool DNA tests, whereas sigmoidoscopy and colonoscopy represent direct methods [78]. The usefulness of the different methods are being

debated [78] but irrespectively of the method, screening for CRC has been shown to be cost-effective and is estimated to save a large number of lives [79].

CRC screening is recommended in the USA and many European countries, but at present not in Sweden. However, a Swedish screening study has recently been initiated, with the aims to study the impact of screening on CRC mortality in Sweden, and to compare the effectiveness of FOBT vs. colonoscopy [80].

Up to now, no reliable serum markers for CRC screening have been identified. Carcinoembryonic antigen (CEA) has proven to be prognostic in early CRC [81] but is not good for screening purposes [81]. At present, CEA is used mainly for postoperative monitoring for recurrent disease [82].

Clinical staging

Disease stage is the strongest predictor of survival for patients with CRC and accurate staging is critical for appropriate patient management [83]. Historically, several classification systems have been used, i.e. the Dukes and Astler-Coller classification systems [84,85]. However, these systems are now considered obsolete and at present, the predominant staging system is the TNM system maintained by the American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC) [83].

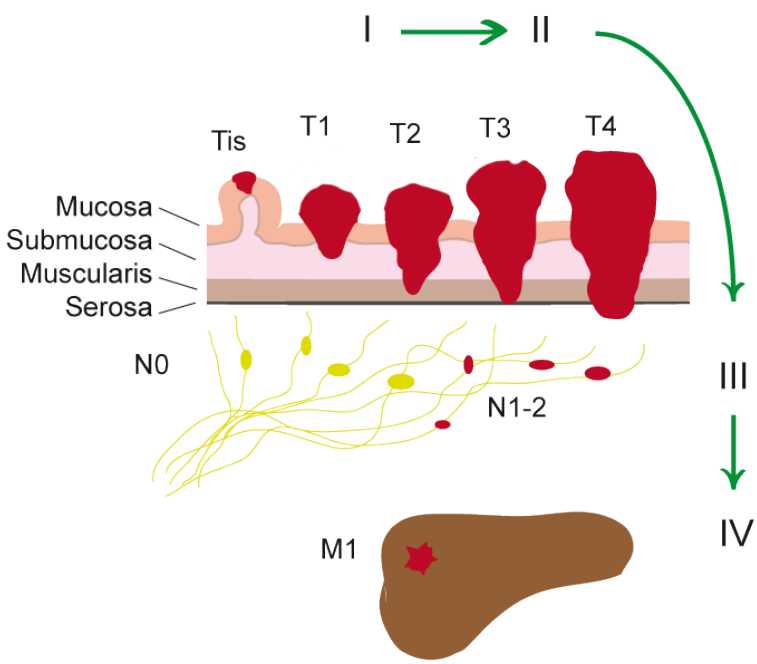


Figure 5. Schematics of the TNM staging system, that codes the extent of the primary tumor (T), regional lymph nodes (N), and distant metastases (M)

The TNM system codes the extent of the primary tumor (T), regional lymph nodes (N), and distant metastases (M) and provides a combined disease stage based on T, N, and M [83,86]. As visualized in Figure 5 and described in detail in Table 1, T-stage refers to the depth of invasion into the intestinal wall and beyond, N-stage denotes the degree of regional lymph node involvement, and M-stage indicates whether the tumour has spread to distant organs, i.e. liver, lungs and peritoneum.

Table 1. The TNM system according to the AJCC cancer staging manual, 7th edition [87].

Primary tumour (T)		**Regional lymph nodes (N)**	
TX	Primary tumour not assessable	NX	Regional lymph nodes not assessable
T0	No evidence of primary tumour	N0	No regional lymph node metastasis
Tis	Carcinoma in situ	N1	Metastasis in 1–3 regional lymph nodes
T1	Tumor invades submucosa	N1a	Metastasis in one regional lymph node
T2	Tumour invades muscularis propria	N1b	Metastasis in 2–3 regional lymph nodes
T3	Tumour invades through muscularis propria	N1c	Tumor deposit(s) in the subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional nodal metastasis
T4a	Tumor penetrates to the surface of the visceral peritoneum	N2	Metastasis in 4 or more regional lymph nodes
T4b	Tumor directly invades or is adherent to other organs	N2a	Metastasis in 4–6 regional lymph nodes
		N2b	Metastasis in 7 or more regional lymph nodes
Distant metastasis (M)			
M0	No distant metastasis		
M1	Distant metastasis		
M1a	Metastasis confined to one organ or site		
M1b	Metastases in more than one organ/site or the peritoneum		

According to the Working Party Report of the World Congress of Gastroenterology in 1991, at least 12 lymph nodes should be dissected and examined by a pathologist after surgical resection of a CRC to achieve an adequate clinical staging [88]. Fewer than 12 examined lymph nodes should be considered a risk factor and adjuvant chemotherapy may be advised [88].

The T, N, and M parameters are further grouped to determine the stage of a tumor and relates to its prognosis, as presented in Table 2.

Table 2. Survival according to TNM stage (AJCC cancer staging manual, 7th edition) [89].

Stage	TNM			5-year survival (%)
I	T1-T2	N0	M0	92
IIA	T3	N0	M0	84
IIB	T4a	N0	M0	76
IIC	T4b	N0	M0	59
IIIA	T1-T2	N1/N1c	M0	83
	T1	N2a	M0	
IIIB	T3-T4a	N1/N1c	M0	64
	T2-T3	N2a	M0	
	T1-T2	N2b	M0	
IIIC	T4a	N2a	M0	32
	T3-T4a	N2b	M0	
	T4b	N1-2	M0	
IVA	any T	any N	M1a	10
IVB	any T	any N	M1b	

Surgery

Surgery is the cornerstone for treatment of CRC, and in most cases involves resection of the primary tumor and regional lymph nodes. Advances in surgery and perioperative care have likely had a significant effect on outcome; with 5-year survival approaching 90% for stages I and II, and >70% for stage III cancers with current adjuvant regimens.

A careful preoperative investigation is essential, and the aims are to confirm the site of the primary tumour, to obtain a histological diagnosis, to rule out synchronous tumours or adenomas, to assess the extent of local and nodal spread (in particular in rectal cancer) and to detect distant metastases [90]. This generally involves a colonoscopy, a CT scan of the thorax and the abdomen and, for rectal cancers, a MRI.

The extent of resection for a colon cancer is based on colonic blood supply. A right hemicolectomy should be performed for cancers of the cecum to the hepatic flexure and includes ligation of the ileocolic, right colic and right branch of the middle colic vessels [91]. Transverse colon cancers require a transverse colectomy. However, an extended right hemicolectomy, with ligation of the ileocolic and middle colic arteries but preservation of the left colic artery, is often preferred because of concerns over tension or inadequate blood supply at the anastomosis [91]. Splenic flexure cancers are managed either by an extended right hemicolectomy or by a left hemicolectomy, with ligation of the inferior mesenteric vessels, depending on the blood supply ascertained after a complete splenic flexure mobilization [91]. Descending and proximal sigmoid colon cancers are

treated with left hemicolectomy, ligating the inferior mesenteric vessels [91]. For mid- and distal sigmoid colon cancers, a sigmoid resection, ligating the left colic artery, is appropriate [17,90]. Anatomic resection based on colonic blood supply assures both adequate margins as well as adequate anastomotic blood supply. The anastomosis can either be stapled or hand-sewn, with similar complication frequencies [91].

Analogously to TME surgery for rectal cancer, the concept of complete mesocolic excision (CME) with central vascular ligation (CVL) has emerged in recent years, whereby the tumour is resected using embryologic tissue planes along with the entire regional mesocolon in an intact peritoneal and fascial lined package [90]. This standardized approach to surgery for colon cancer based on good oncologic principles has been shown to significantly improve survival [92,93], and possibly represents a new era coming.

Surgery for colon cancer is performed in an acute setting in 20-25% of cases [17] which is associated with a significantly increased mortality [94]. The indication for acute surgery can be obstruction, or less commonly perforation or major bleeding [17]. Obstructing cancers are associated with an increased anastomotic leak rate, leading to decreased survival [95]. Generally, obstructing right and transverse colon cancers can be resected with a primary anastomosis, whereas obstructing left-sided cancers most often are managed by resection and a colostomy. In recent years, endoscopic stenting as a bridge to surgery has emerged as an attractive option [91]. Perforated colon cancers have a poor prognosis, regardless of the site of perforation. Perforation may occur at the site of the tumor or proximal to a distal obstruction. In both cases, the area of perforation and the tumor should be resected, and most often a stoma is required [91].

Traditionally, patients with rectal cancer have had a worse prognosis compared to patients with colon cancer. However, rectal cancer management has evolved substantially over the past decades, and this has led to a marked improvement in rectal cancer outcome [96,97], with some countries now reporting a better outcome than for colon cancer [98-100]. This change in scenario can be attributed the cumulative effect of an increased focus on rectal cancer with standardization of surgery, TME [101], use of standardized preoperative staging with MRI [102], and use of neoadjuvant radiotherapy with or without chemotherapy in selected patients [71].

Surgery for rectal cancer is more difficult and demanding than for colon cancer, and should be performed by skilled and dedicated surgeons [103]. The adequate procedure is generally a low anterior resection (LAR), or an abdominoperineal resection (APR) [17,95]. The gold standard for rectal cancer surgery is the TME technique, involving removal of the entire rectal mesentery, including that distal to the tumor, as an intact unit. TME requires precise dissection in an areolar plane along the visceral fascia that envelops the rectum and its mesentery [101].

Tumours of the upper third of the rectum are treated with high ligation of the inferior mesenteric vessels and adequate mobilization of the proximal colon to allow a tension-free anastomosis with a good blood supply [104]. The rectum and its mesorectum are divided 5 cm below the lower end of the tumour, and a primary anastomosis is constructed [104] and can either be hand sewn or stapled. A covering stoma is not usually required [104].

Tumours of the distal and middle thirds of the rectum are managed either by a LAR, or by an APR for the most distal tumours. Since the advent of circular stapling devices, there is no technical lower limit for an anastomosis, however functionality is inferior for distal anastomoses [17,104].

A LAR is performed with the TME-technique, with meticulous dissection and complete mesorectal exicion. The inferior mesenteric artery is ligated, the level of ligature beeing debated extensively [105]. A distal margin of 1-2 cm has proven sufficient [17,90]. After resection, intestinal continuity is restored, commonly with a stapled side-to-end anastomosis [17]. A covering loop ileostomy is recommended, due to the high risk of anastomotic leakage [17]. A Hartmann's procedure, i.e. a resection with closure of the rectal stump and formation of an end colostomy, is an alternative for some patients [17].

An APR consists of synchronous resection of the complete rectum, including the anal canal and the sphincter complex, and the formation of an end colostomy [17]. The abdominal dissection is performed with the TME-technique [17].

Local excision by transanal endoscopic microsurgery (TEM) can be an option for malignant polyps or even T1 tumors located in the rectum for carefully selected patients, such as elderly and fragile patients for whom a more conventional surgical approach would not be appropriate [106,107].

In recent years, there has been a trend towards minimal invasive surgical techniques. For colon cancer, several randomized studies indicate the same oncologic results with laparoscopic surgery as with open surgery [108-110]. For rectal cancer there is evidence that laparoscopic surgery is feasible with less postoperative morbidity and faster recovery, but the oncological outcome remains controversial [111]. Robot assisted surgery for rectal cancer is in its beginning and, consequently, no firm conclusions or recommendations can be given based on the available data [111,112].

The liver and lungs are the most frequent sites (>50%) for distant metastases or recurrent disease, while about 13-25% of patients with recurrent CRC develop synchronous or metachronous peritoneal metastasis. Due to improvements in the surgical and oncological management, cure can be achieved for a selected but increasing number of patients with pulmonary [113], liver [114] and peritoneal metastases [115].

Neoadjuvant and adjuvant treatment

Guided by MRI, advanced stage rectal cancers are treated with neoadjuvant radiotherapy (RT) or chemoradiotherapy (CRT) for downstaging purposes, and to decrease the risk for local recurrence [17,104,116]. The efficacy of different protocols, i.e. short course RT (5x5Gy) and long course RT (2x25Gy), are currently being compared in the Stockholm III trial [117]. Some patients receiving neoadjuvant CRT achieve a complete clinical response (cCR), where no evidence of residual tumor can be found [104]. Recent studies have suggested a "watch and wait policy" in cCR, however this merits further investigation.

Adjuvant chemotherapy is administered after an apparently complete colorectal resection to reduce the risk for recurrence. Most commonly fluorouracil (5-FU) is used, often in combination with leucovorin and oxaliplatin. 5-FU together with leucovorin reduces the risk for recurrence with approximately 30% [72]. With the addition of oxaliplatin, the risk for recurrence is reduced another 20%, however at the cost of significant toxicity [72]. Administration is intravenous, however Capecitabine, a 5-FU prodrug, is an oral alternative with comparable efficacy [72].

At present, adjuvant chemotherapy is offered to patients with TNM stage III disease, i.e. with presence of metastatic spread in the lymph nodes. The potential use of adjuvant therapy in stage II disease has been extensively investigated [118], and might be beneficial in selected cases with high-risk features, such as locally advanced tumours and tumour perforation [72]. Further adverse factors, such as inadequate lymph node sampling, vascular or perineural invasion and poorly differentiated histology are also known to indicate a higher risk of recurrence [119], although the potential benefit of adjuvant chemotherapy is not fully known in patients with tumours displaying these features [72]. Oppositely, MSI is associated with a good prognosis, and may decrease the indication for adjuvant therapy [120]. MSI has further been proposed to indicate resistance to 5-FU based therapies, however recent findings have shown no differences in response between MSS and MSI tumours [57].

The evidence for adjuvant chemotherapy in curatively resected rectal cancer is not as solid as for colon cancer and protocols vary considerably around the world. Shedding some light on this issue, a Cochrane review from 2012 demonstrated significant survival benefits for adjuvant treatment in rectal cancer patients [121].

Also in a palliative setting, fluoropyrimidine-analogs remain the cornerstone, often in combination with oxaliplatin or irinotecan.

In recent years, the development of targeted therapies, e.g. monoclonal antibodies targeting specific molecules implicated in CRC carcinogenesis, have changed the scene somewhat:

The angiogenesis inhibitor bevacizumab is a monoclonal antibody targeting the vascular endothelial growth factor (VEGF) and is generally used in combination

with chemotherapy for patients with advanced CRC [72]. Bevacizumab has a documented effect on survival in patients with metastatic CRC (mCRC) [122], but has not proven to be useful as adjuvant treatment [122].

Cetuximab and panitumumab are monoclonal antibodies targeting the epidermal growth factor receptor (EGFR). Cetuximab is used in combination with chemotherapy for mCRC in both first and second line settings, or as third line monotherapy [122]. Panitumumab have similar indications. The clinical effect of EGFR inhibitors has been extensively studied, with mixed results [122]. Adjuvant use is unsupported [122].

Importantly, EGFR inhibitors have shown clinical efficacy only in tumors that are KRAS wild type, and not in those with KRAS activating mutation [123-127]. Therefore, KRAS mutation status is routinely tested prior to initiation of anti-EGFR therapy. Similarly, EGFR inhibitors are most effective in tumors that are BRAF wild type [122].

Investigative markers

Advances in molecular technology have resulted in the discovery of many putative biomarkers relevant to CRC, but nearly all are still in the discovery phase waiting to undergo clinical validation.

Cyclin D1

The cyclin D1 proto-oncogene belongs to the highly conserved cyclin family and plays a key role in cell cycle control, particularly in the transition from G1 to S phase [128,129]. Together with cyclin dependent kinase 4 and 6 (CDK4 and CDK6), cyclin D1 forms an active complex that promotes cell cycle progression by phosphorylation and, hence, inactivation of the retinoblastoma protein (pRb) [128,129]. Recent findings have also shown that cyclin D1 functions as a transcriptional modulator [128,130].

Cyclin D1 is considered important for the development and progression of several cancers [128,129]. Further, overexpression and amplification of cyclin D1 has been linked to the development of endocrine resistance in breast cancer in vitro and in vivo [128,131]. Increased levels of cyclin D1 may result as a consequence of gene amplification or from defective regulation at the post-translational level [128,132]. The oncogenic effect of cyclin D1 is due to enhancement of several processes during malignant cell transformation, such as abnormal growth, angiogenesis, and resistance to apoptosis [133]. Cyclin D1 is a target of the Wnt-pathway, and it has been suggested that cyclin D1 activation secondary to APC or beta-catenin mutation is implicated in CRC carcinogenesis [134,135]. Further, cyclin D1 is also commonly activated through the Ras/Raf/MEK/MAPK pathway [130].

Cyclin D1 is frequently overexpressed in CRC [136-144], however the relation between cyclin D1 expression and clinical outcome in CRC is uncertain. A summary of published studies on the prognostic value of cyclin D1 in CRC is presented in Table 3.

Table 3. Summary of studies on the clinicopathological correlates and prognostic value of cyclin D1 expression in CRC.

Study	Year	n	Significant associations/main findings
Maeda et al [140]	1997	101	Advanced T-stage. Poor prognosis.
Bahnassy et al [136]	2004	60	Advanced tumour size, T-stage and N-stage. Poor prognosis.
Holland et al [138]	2001	126	Low differentiation and proximal tumour location. Nuclear expression associated with p21 expression. Good prognosis.
Ogino et al [142]	2009	602	MSI-high, CIMP-high and BRAF-mutation. Expression of p21 and p27. Good prognosis.
Jang et al [139]	2012	220	Good prognosis, overall and in adjuvantly treated patients.
MacKay et al [145]	2002	249	Expression of p21 and p27. Old age. Not independently prognostic.
Knösel et al [146]	2005	270	Not prognostic
Formentini et al [147]	2012	140	Not prognostic
Hislka et al [148]	2005	363	Not independently prognostic
Kouraklis et al [149]	2006	111	Not independently prognostic
Lyall et al [150]	2006	90	Not independently prognostic
Schmitz et al [151]	2007	135	Not independently prognostic
Theocharis et al [152]	2007	86	Not independently prognostic
Saridaki et al [153]	2010	144	Not independently prognostic
Mao et al [154]	2011	169	Not independently prognostic

Beta-catenin

Beta-catenin is a membrane-associated protein that plays a dual role in the cell. It has an essential function in the regulation of cellular adhesion, and further serves as the major mediator of the Wnt-signaling pathway, which has a crucial role in embryonic development and tissue regeneration [155]. Wnt-signaling has further been implied to be involved in energy metabolism [156,157]. Mutations in the Wnt-pathway further have an important role in the development of CRC [155,158].

Beta-catenin forms a complex with kinases GSK3 and CK1, and tumour suppressor proteins APC and Axin. When Wnt receptors are not engaged, kinases in the complex phosphorylate beta-catenin, thus targeting the latter for rapid

destruction. When receptors are activated by Wnt ligands, the intrinsic kinase activity of the complex is inhibited. As a consequence, stable nonphosphorylated beta-catenin accumulates and makes its way into the nucleus, where it coactivates transcription of various target genes, such as cyclin D1 and c-Myc and, hence, regulates cell proliferation and apoptosis [155,159].

Table 4. Summary of studies on the clinicopathological correlates and prognostic value of beta-catenin expression in CRC.

Study	Year	n	Significant associations/main findings
Chen et al [160]	2013	3665	Meta-analysis. Nuclear expression associated with advanced disease stage and poor prognosis.
Andras et al [161]	2012	100	Loss of membranous expression associated with poor prognosis
Stanczak et al [162]	2011	66	Nuclear expression associated with a poor prognosis
Matsuoka et al [163]	2011	156	Loss of membranous expression in the invasive front associated with poor prognosis
Ougolkov et al [164]	2002	202	Nuclear expression in the invasive front associated with advanced disease stage and recurrent disease
Toth et al [165]	2012	79	Nuclear expression associated with distant metastasis
Sun et al [166]	2011	67	Nuclear expression associated with advanced T- and M-stage
Chen et al [167]	2008	96	Nuclear expression associated with advanced disease stage and low differentiation grade. Not independently prognostic.
Bravou et al [168]	2006	125	Nuclear expression associated with advanced disease stage
Fernebro et al [169]	2004	269	Loss of membranous or cytoplasmic expression associated with metastatic disease
Morikawa et al [170]	2011	995	Nuclear expression associated with a favorable prognosis in obese patients
Pancione et al [171]	2010	141	Expression associated with a favorable prognosis
Jang et al [139]	2012	220	Not independently prognostic
Mårtensson et al [172]	2007	67	Not independently prognostic
Togo et al [173]	2008	183	Not prognostic

Thus, translocation of beta-catenin to the nucleus is an indicator of an active Wnt-signaling pathway [160]. Nuclear beta-catenin overexpression is common in CRC and has been linked to CIN [172,174]. Mutation in APC is the predominant factor causing aberrant Wnt-signaling, however mutation in beta-catenin and Axin also occur [159,175]. The clinical significance of altered beta-catenin expression in CRC is controversial, and previous results are conflicting [139,161-173,176-179]. However, a recent meta-study concluded that nuclear beta-catenin overexpression is independently associated with a poor prognosis [160]. A summary of published

studies on the prognostic value of beta-catenin expression in CRC is presented in Table 4. Of note, beta-catenin staining evaluation protocols in these studies are quite heterogeneous.

MSI

As previously described, MSI in sporadic CRC is caused by silencing of MMR genes though hypermethylation [53], due to the CIMP phenotype [53]. MSI tumours are associated with female sex, proximal location, low differentiation grade and mucinous histology [53]. Further, MSI is, without controversy, associated with a good prognosis [55,180].

MSI has been shown to indicate resistance to various chemotherapeutic agents, including 5-FU, in vitro [56]. However, clinical studies on the use of MSI as a predictive marker of chemotherapy response are conflicting [55,57,180-182].

KRAS

KRAS is a membrane-associated GTPase protein, in humans encoded by the v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) proto-oncogene, with a central role in many cellular signal transduction pathways connecting extracellular signals with nuclear transcription factors [183,184]. Inactive KRAS is bound to GDP, which is exchanged with GTP upon activation by cell surface receptors, such as EGFR, leading to subsequent downstream signal transduction. This is transient however, as GTP is converted to GDP by intrinsic enzymatic activity and KRAS turns itself off, acting as a self limiting molecular on/off switch. Mutated KRAS, predominantly in codons 12 and 13, remains in the active GTP-bound state and regulation of downstream signaling is lost [49,183,185]. Approximately 40% of all CRCs have an activating KRAS mutation [49,183,184,186-189]. The mutational incidences for ACFs, adenomas and carcinomas are similar, indicating that KRAS mutation represents an early step in CRC carcinogenesis [190].

KRAS acts downstream of EGFR in the Ras/Raf/MEK/MAPK pathway, and KRAS mutation has proven to be predictive of resistance to EGFR-inhibiting therapies [123-127]. Thus, KRAS mutation analysis has become clinical routine before considering administration of such drugs.

Numerous studies have investigated the relationship between KRAS mutation status and survival from CRC, with divergent results, however the majority have demonstrated an association between KRAS mutation and poor prognosis [67,123,189,191-195]. An overview is presented below in Table 5. Notably, while most studies did not consider specific mutations, accumulating evidence indicates that specific codon 12 and 13 mutations have a different impact on the functionality of the KRAS protein, and, hence, its impact on clinical outcome in CRC patients [191,196,197].

Table 5. Summary of studies on the prognostic value of KRAS mutation in CRC.

Study	Year	n	Stage	Significant associations/main findings
Richman et al [198]	2009	711	IV	Any KRAS mutation associated with poor prognosis
Nash et al [192]	2010	532	I-IV	Any KRAS mutation associated with poor prognosis
Farina-Sarasqueta et al [199]	2010	364	II-III	Any KRAS mutation associated with poor prognosis
Hutchins et al [193]	2011	1913	II	Any KRAS mutation associated with poor prognosis
Phipps et al [194]	2013	1989	I-IV	Any KRAS mutation associated with poor prognosis
Samovitz et al [197]	2000	1413	I-IV	Codon 12 mutation associated with proximal tumour and advanced tumour stage. G13A mutation associated with poor prognosis.
Andreyev et al [189]	2001	3439	I-IV	G12V mutation associated with poor prognosis
Bazan et al [191]	2002	160	I-III	Codon 13 mutation associated with advanced disease stage and poor prognosis. Codon 12 associated with mucinous histology.
Imamura et al [200]	2012	1075	I-IV	Codon 12 mutation associated with poor prognosis
Yokota et al [201]	2011	229	IV	Not independently prognostic
Mouradov et al [202]	2013	375	II-III	Not prognostic
Lee et al [203]	2008	134	I-IV	Not prognostic
Wang et al [204]	2003	396	II	Not prognostic
Ogino et al [67]	2009	649	I-IV	Not prognostic
Roth et al [195]	2010	1404	II-III	Not prognostic

BRAF

V-raf murine sarcoma viral oncogene homolog B (BRAF) is a proto-oncogene, encoding for the serine/threonine protein kinase BRAF [205]. Acting downstream of KRAS in the Ras/Raf/MEK/MAPK pathway, BRAF plays an important role in the regulation of signal transduction between the extracellular environment and the nucleus [184].

BRAF mutation, predominantly a V600E substitution, results in constitutive activation, and has been reported in CRC at a frequency of 5-18% [184,205,206]. Generally, KRAS and BRAF mutations are mutually exclusive [207].

Further, BRAF mutation is closely associated with CIMP and MSI [205], and has been reported to indicate resistance to anti-EGFR therapies [208-210]. Despite the association with MSI, which is associated with a good prognosis [55], BRAF mutation appears to be associated with a poor prognosis in CRC [195,199,210-

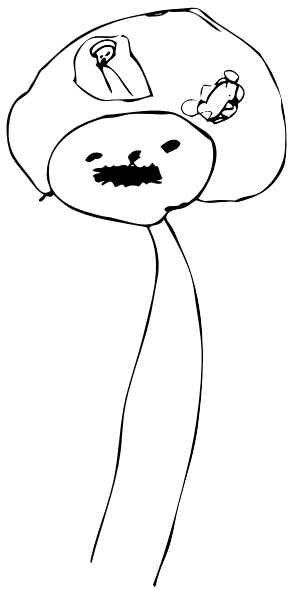
215]. However, the association between BRAF mutation and poor prognosis has been reported to be evident only in combination with MSS tumours, which might explain the paradox [199,211-213]. Moreover, BRAF mutation is considered a marker for the "serrated pathway", which involves the progression of a serrated lesion to cancer [216].

EGFR

The epidermal growth factor receptor (EGFR) is a transmembrane tyrosine kinase receptor with an important role in the Ras/Raf/MEK/MAPK and PI3/PTEN/AKT signaling cascades [217]. Activation by the main ligands epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α) leads to intracellular signal transduction, gene activation and stimulation of cell cycle progression [218]. Dysregulation of EGFR function contributes to the growth and survival of cancer cells, and is observed in a variety of malignancies [219].

EGFR overexpression and gene copy number (GCN) alterations are commonly seen in CRC [218,220-222]. A high level of EGFR protein expression in CRC has been correlated with advanced stage disease [218,222,223] and a poor prognosis [220,223-226], whereas an increased EGFR gene copy number (GCN) has been associated with clinical response to anti-EGFR treatment [227-229].

The correlation between EGFR protein expression and GCN is unclear, and the results from previous studies are conflicting [230-235]. Further controversies exist about the validity of the different detection methods, i.e. immunohistochemical evaluation of EGFR protein expression vs. EGFR gene copy number (GCN) measured by different in situ hybridization techniques [219,229] and lack of standardization in scoring systems and cutoffs adds further debate [219,236].



The present investigation

General aims

The main focus of this thesis was to investigate the prognostic and treatment predictive value of a number of putative biomarkers in CRC, with a special focus on sex differences. Further, the aim was to study the associations between the investigated biomarkers and clinicopathological and molecular characteristics.

Methods

Patients

The Malmö Diet and Cancer Study (MDCS) is a prospective population-based study designed to investigate the impact of diet and other lifestyle factors on the risk of developing cancer. Between 1991 and 1996, 18326 women (60.2%) and 12120 (39.8%) men were enrolled, with a total of 30446 participants (from a background population of 74,138). Subjects were aged between 44-74 years [237].

Until 31 Dec 2008, 626 incident cases of CRC had been registered in the MDCS. Cases were identified from the Swedish Cancer Registry up until 31 Dec 2007, and from The Southern Swedish Regional Tumour Registry for the period of 1 Jan - 31 Dec 2008. All tumours with available slides or paraffin blocks were histopathologically re-evaluated on haematoxylin and eosin stained slides. Histopathological, clinical and treatment data were obtained from the clinical and/or pathology records. TNM staging was performed according to the American Joint Committee on Cancer (AJCC). Information on vital status and cause of death was obtained from the Swedish Cause of Death Registry up until 31 Dec 2009. Follow-up started at date of diagnosis and ended at death, emigration or 31 Dec 2009, whichever came first. None of the CRC cases registered until 31 Dec 2008 was lost due to emigration during follow-up. Median follow-up time was 3.35 years (range 0–17.69) for the full cohort (n = 626) and 6.05 years (range 1.03-17.69) for patients alive (n = 344). Ethical permission was obtained from the

Ethics Committee at Lund University for the MDCS (Ref. 51/90), and the present study (Ref. 530/2008).

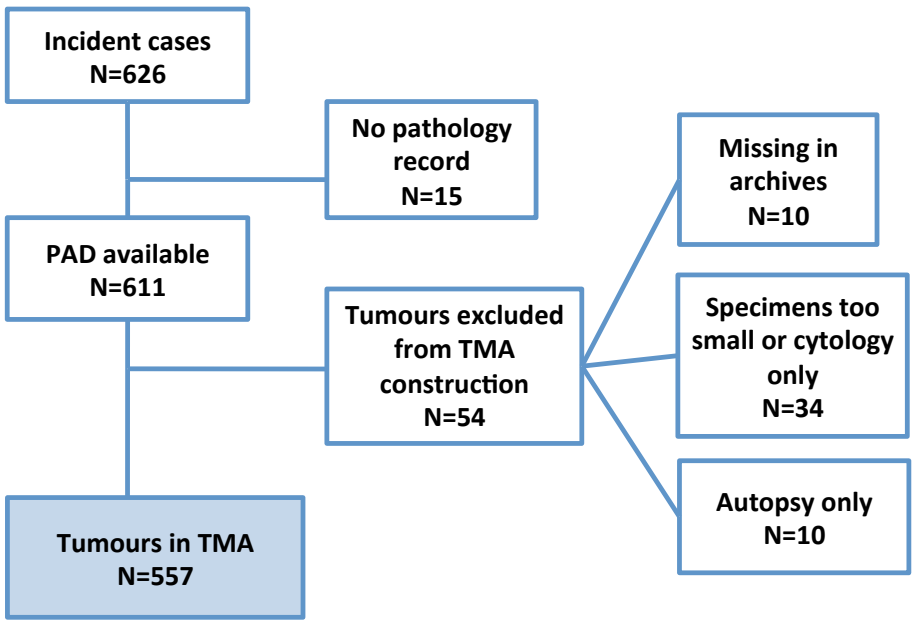


Figure 6. Incident CRC in the Malmö Diet and Cancer Study, as of 31 December 2008.

Tissue microarray construction

Cases with an insufficient amount of tumour material were excluded, whereby a total number of 557 (89.0%) tumours were suitable for tissue microarray (TMA) construction. Areas representative of cancer were marked on haematoxylin and eosin stained slides and TMAs were constructed.

In brief, two 1.0 mm cores were taken from each tumor and mounted in a new recipient block using a semi-automated arraying device (TMArrayer; Pathology Devices, Westminster, MD, USA). Four µm sections from this block were subsequently cut using a microtome and mounted on glass slides (Figure 7).

The TMA technique is a well-established research tool that enables high-throughput simultaneous analysis of multiple tissue specimens. Further, utilization of limited tissue material is optimized [238].

Tumor heterogeneity may represent a technical limitation. However, it should be pointed out that conventional whole tissue sections also only represent selected

areas of a tumour, and the TMA technique may provide even better prognostic information than large tissue sections [239]. Furthermore, TMA is used for population-level research, and not clinical diagnosis of individual cases and, thus, tumor heterogeneity generally is not a limitation [240,241].

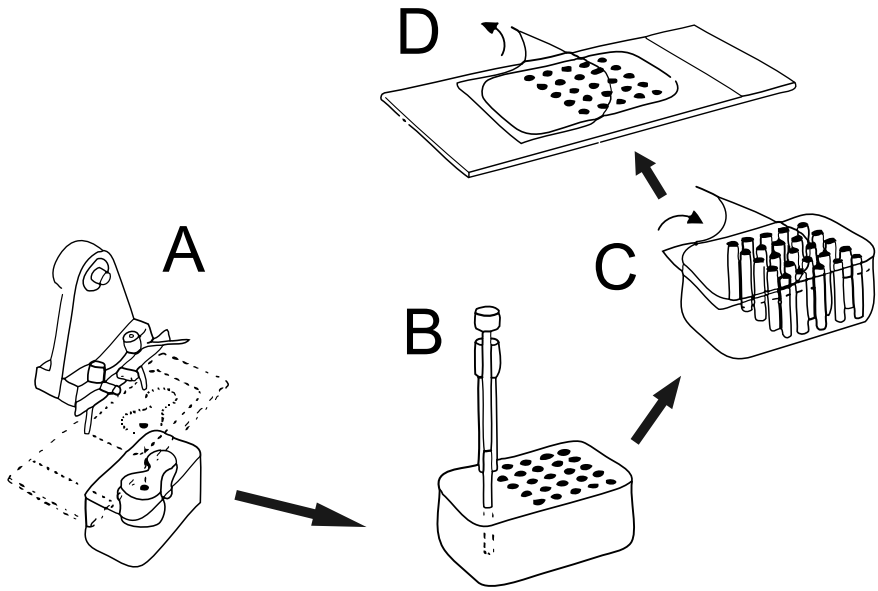


Figure 7. The TMA technique. A tissue core biopsy is punched from a preselected region of the donor block (A). The tissue core is mounted in a recipient block (B). Four µm sections from the recipient block are cut (C) and mounted on glass slides (D). Used with permission by John Wiley & Sons Ltd [240].

Immunohistochemistry

The fundamental concept behind immunohistochemistry (IHC) is the demonstration of antigens within tissue sections by means of specific antibodies. The antigen–antibody interaction can then be visualized, commonly by using antibodies labeled with an enzyme, such as peroxidase, that catalyzes a colour-producing reaction.

In this study, 4 µm TMA sections were deparaffinized, rehydrated and pretreated with the heat induced epitope retrieval method (HIER), using the PT-Link system (Dako, Glostrup, Denmark). Pretreatment, or antigen retrieval, is required to unmask hidden epitopes due to formation of methylene bridges caused by fixation [242]. TMA sections were then stained (Autostainer Plus; Dako) with the monoclonal antibodies described in Table 6.

Table 6. Antibodies used in papers I-IV

Marker	Manufacturer	Clone	Dilution	Paper
Cyclin D1	Dako	DSC-6	1:50	I-IV
beta-catenin	BD Pharmingen	14/Beta-Catenin	1:5000	II-IV
p21	Dako	SX118	1:25	II-IV
p27	Dako	SX53G8	1:100	II-IV
p53	Dako	DO-7	1:100	II-IV
MLH1	Dako	ES05	1:100	II-IV
PMS2	BD Pharmingen	A16-4	1:300	II-IV
MSH2	Calbiochem	FE11	1:100	II-IV
MSH6	Epitomics	EPR3945	1:100	II-IV
EGFR	Zymed	31G7	1:25	IV
EGFR	Ventana	3C6	dispensed	IV

Staining was evaluated by two independent observers, blinded to data on clinical outome. Any scoring differences were discussed in order to reach consensus.

The different scoring models are described in each paper.

Pyrosequencing

Pyrosequencing is a DNA sequencing technique based on the sequencing by synthesis principle. Made simple, nucleotides are sequentially added to a DNA-template. If the nucleotide is complementary, it binds to the DNA and through a cascade of enzymatic reactions, visible light is generated proportional to the number of incorporated nucleotides, and as the added nucleotide is known, and the amount of light emitted can be measured, the sequence of the template can be determined [243]. A sample pyrogram is presented in Figure 8.

In paper III, the PyroMark Q24 system (Qiagen GmbH, Hilden, Germany) was used for pyrosequencing analysis of KRAS and BRAF mutations in DNA from 1 mm formalin-fixed, paraffin-embedded tumour tissue cores taken from areas with >90% tumour cells. In brief, genomic DNA was extracted from tumour tissue using QIAamp MinElute spin columns (Qiagen) and DNA regions of interest were PCR-amplified (Veriti 96-Well Fast Thermal Cycler, Applied Biosystems Inc., Foster City, CA, USA). KRAS codons 12 and 13 were analyzed using Therascreen KRAS Pyro Kit (Qiagen). Analysis of BRAF mutation hotspots in codons 600 and 601 was performed using previously published PCR primers [198] and a novel BRAF sequencing primer (5′-TGATTTTGGTCTAGCTACA-3′), which was designed using the PyroMark Assay Design 2.0 software (Qiagen). All samples with a potential low-level mutation were reanalyzed.

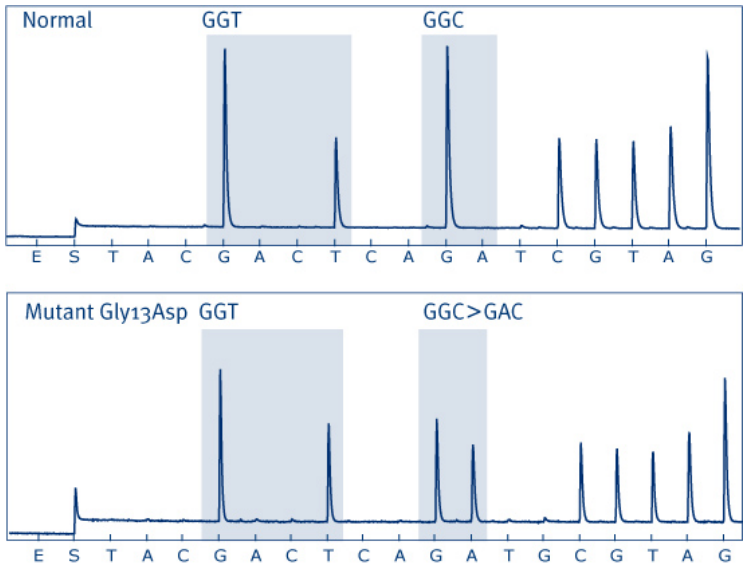


Figure 8. A pyrogram demonstrating a KRAS wild-type genotype (top) and a G13D (gly13→asp13) mutation in codon 13 (bottom).

In situ hybridization

In situ hybridization (ISH) techniques use a labeled strand of complementary DNA or RNA, i.e. probe, to localize a specific DNA or RNA sequence in a tissue sample, and can be used to detect chromosomal imbalances. The method applied in paper IV, brightfield double in situ hybridization (BDISH), uses two separate probes on a single slide: an EGFR gene (localized in chromosome 7) probe, resulting in a black signal, and a chromosome 7 centromere (CEN7) probe, resulting in a red signal. Thus, the level of EGFR gene amplification can be assessed by calculating EGFR/CEN7 ratios [244].

In specific, automated BDISH was performed on Ventana Benchmark Ultra (Ventana Medical Systems). Ultraview Inform Chromosome 7 DIG probe RUO and EGFR DNP Probe RUO (Ventana Medical Systems) were visualized on the same slide. Assay conditions were modified to obtain optimal results. The whole assay procedure (deparaffinization, pretreatment, hybridization, stringency wash, signal detection and counterstaining) was fully automated. Both probes were denatured at 80 °C for 8 min and hybridized at 44 °C for 6 h. Stringency washes were performed at 72˚C for 8 min. The silver signal for EGFR was revealed by sequential silver reactions. The signal of the centromere was visualized with the RedISH Naphtol reaction. The tissues were counterstained with Hematoxylin II and Bluing Reagent. ISH was scored with the use of a brightfield microscope (Olympus BX45) with 40x and 60x objective. EGFR amplification was considered

to be present when the EGFR/CEN7 ratio was ≥2 within 20 tumour cell nuclei. Ratios between 2 and 5 were considered as low-level amplification and >5 as high-level amplification. Polysomy was defined as increased numbers of EGFR gene copies as well as CEN7 signals with a ratio > 2.

Statistics

Associations between the investigated biomarkers and clinicopathological factors were explored by Spearman's rank correlation in paper I and by Pearson's Chi-squared test in paper II-IV. Kruskal-Wallis or Mann-Whitney U test was applied for continuous variables. Kaplan-Meier analysis and log-rank test were performed to illustrate differences in survival. Further, Cox proportional hazards regression was used for estimation of hazard ratios (HR) for death from CRC in both uni- and multivariable analysis. A backward conditional method was used for variable selection in multivariable models. The interaction between investigative factors and sex in paper I and adjuvant treatment in paper IV was explored by a Cox model including the interaction variable. All tests were two-sided. A p-value of 0.05 was considered significant. All statistical analyses were performed using IBM SPSS Statistics version 20.0.

Paper I

Aims

The main objective of paper I was to examine the association of nuclear cyclin D1 expression with established clinicopathological characteristics and survival from CRC, in the full cohort and in subgroups according to gender. Further, the distribution of clinicopathological characteristics and treatment given was explored.

Summary of results

The distribution of patient and tumour characteristics, as well as treatment given, did not differ significantly between subgroups according to gender. There was no significant sex-related difference in cancer-specific survival (CSS), neither in the entire cohort nor in patients with stage IV disease at diagnosis. IHC nuclear cyclin D1 expression was evaluated as intensity and fraction, and could be assessed in 527 tumours. Whereas 105 (19.9%) tumours did not express cyclin D1, the remaining 422 (80.1%) expressed cyclin D1 in various fractions and intensities. The intensity, but not the fraction, of nuclear cyclin D1 was significantly lower in male CRC (p=0.018).

Further, the association of cyclin D1 expression with clinicopathological characteristics was examined. In the full cohort, cyclin D1 fraction was associated with age (r=0.101, p=0.020), and inversely associated with T-stage (r=-0.105, p= 0.018), N-stage (r=-0.114, p=0.012), M-stage (r=-0.091, p=0.039) and vascular invasion (r=-0.121, p=0.034). In female patients, cyclin D1 fraction was inversely associated with vascular invasion (r=-0.175, p=0.026). Finally, in male patients, cyclin D1 fraction, as well as intensity, was inversely associated with N-stage ((r=-0.134, p=0.041); (r=-0.153, p=0.020)), and M-stage ((r=-0.143, p=0.024); (r=-0.161, p=0.011)).

Proceeding with survival analysis, we found that cyclin D1 expression, dichotomized as no expression vs. any expression, was significantly associated with an improved CSS in the full cohort (HR=0.69; 95% CI=0.49-0.96) and in

men (HR=0.48; 95% CI=0.31-0.74), but not in women. However, these associations did not remain significant when adjusting for age, gender, TNM-stage, differentiation grade and vascular invasion. Cox interaction analysis confirmed a significant interaction between cyclin D1 expression and gender (p=0.024), however significance was not retained in multivariable analysis. Kaplan-Meier analysis in combined subgroups according to gender and cyclin D1 expression revealed a significantly reduced CSS for men with cyclin D1-negative tumours compared to men with cyclin D1-positive tumours, and all women, irrespective of cyclin D1 expression. Stratifying further for disease stage, this association was not evident in stage I-II but remained significant in stage III-IV.

Finally, we looked at the potential impact of cyclin D1 expression on response to adjuvant treatment in curatively treated patients with stage III disease, but no significant correlations were observed.

Discussion

In this paper, we demonstrate that cyclin D1 expression is associated with a favorable prognosis in male, but not in female, CRC. This association was however not independent of established prognostic factors. We further observed an inverse association of cyclin D1 expression with TNM-stage and vascular invasion, both representing established adverse prognostic factors, which may explain the lack of independent significance.

Several previous studies have investigated the role of cyclin D1 expression in CRC, with divergent results [136,138-140,142,145-154]. However, several recent studies link cyclin D1 expression to a good prognosis [138,139,142], further validating our findings.

Despite obviously carrying oncogenic properties [133], tumour-specific cyclin D1 expression seems to indicate a good prognosis. However, CRC carcinogenesis is complex, and the accumulation of genetic and epigenetic events can follow different pathways. Consequently, a tumour bypassing cyclin D1 activation may develop an even more aggressive phenotype. Analogously, MSI is also associated with a good prognosis [55]. Moreover, associations between cyclin D1 expression and a good prognosis have also been described in lung-, breast-, and bladder cancer [245-247].

To our knowledge, the sex-related difference in the prognostic value of cyclin D1 has not been reported previously, and neither has the observation of a lower nuclear cyclin D1 intensity in male compared to female CRC.

Several previously observed sex-related differences in CRC epidemiology and biology indicate a hormonal influence, such as the incidence generally being

higher in men [1,3], and women more often having proximal tumours, characterized by MSI and CIMP [63,67]. Further, postmenopausal HRT with estrogen and progestin have proven to significantly reduce CRC incidence rates in several studies[34,248], though results on the CRC-protective effect of estrogen only treatment are conflicting [35,249]. However, the reduction in CRC risk associated with estrogen and progestin use has been reported to be confined to patients with MSS tumours [248]. Epidemiological data further indicate that oral contraceptives (OC) users have a reduced risk of colorectal cancer [250].

Cyclin D1 is an important mediator of estrogen signaling, either by direct interaction or by activation through the Wnt-pathway [130,251,252]. Estrogen receptors, predominantly ERβ, are commonly expressed in CRC [253] and are considered to have a prominent role in the biological mechanisms of sex steroid action on colorectal tissue [254].

Of note, the sex-related prognostic effect of cyclin D1 was evident in stage III-IV, but not in stage I-II, disease. We have no obvious explanation to this observation, however, a not too far-fetched speculation is that cyclin D1 expressing tumours represent a less aggressive phenotype, even when being in a disseminated state.

Cyclin D1 expression has previously been linked to MSI and CIMP [141,142]. We did not have MSI data at the time of publishing paper I, but in paper II we report a strong association between cyclin D1 expression and MSI. Thus, MSI, indicating a good prognosis, may be a confounding factor. However, it is reasonable to assume that cyclin D1 is involved in the development of MSI.

A common polymorphism of the cyclin D1 gene, A870G, appears to be a low-penetrant risk factor for CRC [255], and further seems to be associated with HRT-associated CRC risk reduction [256]. Hence, it would be of interest to study the associations of different cyclin D1 genotypes and risk of CRC, overall and according to tumour-specific cyclin D1 expression, in the MDCS.

The distribution of patient and tumour characteristics were in line with the expected, except the low frequency of acute surgery (8.7%). Generally, the reported frequency of acute surgery in CRC is approximately 25% [257,258]. One explanation for this discrepancy might be a high health awareness among the study participants. Further, information on the type of surgery was missing for 5.1% of patients.

In conclusion, we here demonstrate that cyclin D1 expression is a favourable, however not independent, prognostic factor in male but not in female CRC. This further confirms the involvement of cyclin D1 in CRC carcinogenesis and adds weight to the accumulating evidence that CRC is a sex hormone-dependent disease.

Paper II

Aims

In paper II, we moved the focus upstream of cyclin D1, to the Wnt-pathway mediator beta-catenin, and to microsatellite instability (MSI) status, which also have been reported to interact with cyclin D1 [141]. We further looked at cell cycle regulators p21 and p27, and tumour suppressor p53. The prognostic and treatment predictive significance of beta-catenin expression and MSI was explored, and correlations between investigative and clinicopathological factors were examined.

Summary of results

MSI screening status was defined as MSI for tumours lacking nuclear IHC staining for MLH1, PMS2, MSH2 or MSH6, and MSS for tumours expressing all four MMR proteins. Out of 515 assessable tumours, 438 (85%) were MSS, and 77 (15%) were MSI. MSI was positively associated with older age ($p=0.003$), female sex ($p=0.021$), proximal tumour location ($p<0.001$), low differentiation grade ($p=0.003$), and inversely associated with N-stage ($p=0.017$) and M-stage ($p=0.010$). Further, there was a positive correlation between MSI and expression of cyclin D1 ($p<0.001$ for fraction and intensity), and p21 ($p=0.002$ for fraction and $p<0.001$ for intensity), and an inverse correlation between MSI and expression of p53 ($p<0.001$) and p27 ($p=0.003$ for fraction and $p<0.001$ for intensity).

Immunohistochemical beta-catenin expression was evaluated according to a previously described protocol [259], whereby membranous staining was denoted as 0 (present) or 1 (absent), cytoplasmic staining intensity as 0-2, and nuclear staining intensity as 0-2. The total score ranging from 0 (corresponding to membranous staining only, as in normal colonic mucosa) to 5 (tumours with strong nuclear and cytoplasmic staining) was either dichotomized (0-2 / 3-5) or trichotomized (0-1 / 2-3 / 4-5). A high beta-catenin score was associated with distal tumour location ($p<0.001$), low T-stage ($p=0.039$) and intermediate or high differentiation grade ($p=0.016$), and further with expression of p53 ($p<0.001$), cyclin D1 ($p=0.001$ for fraction) and p27 ($p<0.001$ for fraction and intensity). An

inverse correlation was observed between MSI and beta-catenin overexpression ($p<0.001$).

In survival analysis, MSI was associated with an improved CSS in the full cohort (HR=0.50; 95% CI=0.29-0.84) and in stage III-IV (HR=0.46; 95% CI=0.23-0.95), but not in stage I-II, disease. These associations remained significant when adjusting for age, TNM-stage, differentiation grade and vascular invasion (HR=0.46; 95% CI=0.25-0.84 for the entire cohort and HR=0.33; 95% CI=0.14-0.78 for stage III-IV disease).

A high beta-catenin score also correlated with a prolonged CSS in the full cohort and in stage III-IV, but not in stage I-II, disease. These associations were however weaker than for MSI in that significance was only reached in adjusted analysis (HR=0.70; 95% CI=0.51-0.97 for the entire cohort and HR=0.67; 95% CI=0.46-0.97 for stage III-IV disease), and not in unadjusted analysis.

MSI and beta-catenin overexpression did not predict response to adjuvant treatment in stage III disease, and no sex-related differences in the prognostic value of MSI and beta-catenin overexpression were observed.

Discussion

Here, we have shown that MSI independently predicts a prolonged survival from CRC, both in the full cohort and in stage III-IV disease. The majority of previous studies link MSI to a good prognosis [55,180], and our findings further confirm this association. MSI represents a distinctively different pathway of carcinogenesis than CIN [120], which in turn seems to be associated with a poor prognosis [50]. Thus, it is unclear whether the good prognosis associated with MSI is independent of, or merely reflects the absence of CIN.

Of note, MSI was prognostic in stage III-IV, and not in stage I-II, disease. These findings differ somewhat from previous studies in which MSI has proven to be an independent prognostic factor also in stage II CRC [202,260-262]. Despite being associated with a good prognosis, MSI tumours are known to display several adverse prognostic features, such as advanced T-stage and low differentiation grade[263]. Although the causality behind this discrepancy in clinicopathological characteristics and survival is unknown, the favorable phenotype associated with MSI could explain why MSI is associated with an improved survival even in metastatic disease. Interestingly, hepatic metastases from CRC rarely display MSI [264].

As the median patient age in the investigated cohort is 71.4 years, most cases with MSI are likely sporadic tumours caused by MLH1 hypermethylation / CIMP. However, CIMP does not invariably result in MSI, and although CIMP-high /

MSS tumours represent a minority [59], it would be of great interest to investigate CIMP status of the tumours in this cohort.

As previously mentioned, MSI has been suggested to indicate resistance to chemotherapy, although clinical data are somewhat inconsistent [55,57,180-182]. In our study, we did not observe any influence of MSI on treatment response in stage III disease. It should however be pointed out that the subgroups available for analysis were rather small, thus limiting the analysis.

We further demonstrated significant associations between MSI and clinicopathological characteristics, such as old age, female sex, proximal tumour location and low differentiation grade. These associations are all in line with the expected [53], therefore further validating our data. Molecular correlates with MSI were also in concordance with previous studies [141,265].

Beta-catenin overexpression was also associated with a good prognosis, both in the full cohort and in stage III-IV disease, however not as significantly as for MSI. Notably, these associations were only significant in multivariable, and not in univariable, analysis, which might indicate a strong interaction between covariates. Previous studies on the prognostic value of beta-catenin in CRC show divergent results [139,160-166,168-173,176], however a recent meta-analysis concluded that nuclear beta-catenin overexpression indicates a poor prognosis [160]. The discrepancy in results regarding the prognostic value of beta-catenin overexpression may be attributed to several factors. Firstly, the intracellular localization of beta-catenin in CRC, as evaluated by IHC, has shown wide variability in terms of proportions of nuclear, cytoplasmic, or membranous staining [266,267]. Furthermore, different IHC staining methods and scoring protocols have been used, and, lastly, as it has been suggested that aberrant beta-catenin activation is manifested in a heterogeneous intratumoural distribution of nuclear beta-catenin expression [268,269], TMA-based analyses may not be optimal.

As with MSI, significant clinicopathological and molecular correlates of beta-catenin overexpression were in line with the expected [139,170,172]. Moreover, the finding of MSI being inversely associated with beta-catenin overexpression is also expected, as beta-catenin overexpression, caused by aberrant APC activation or by mutation, is associated with CIN [46,174,270], thus representing a distinctively different pathway to CRC carcinogenesis than MSI.

Both MSI and beta-catenin overexpression were associated with an improved survival, which might be perceived as contradictory. However, beta-catenin overexpression is not exclusive for CIN [155,175], and, furthermore, CIN and MSI are not mutually exclusive pathways [265].

In conclusion, the results in this paper demonstrate that MSI is an independent prognostic factor in CRC, but not in localized disease, and does not predict

response to adjuvant chemotherapy. Despite the inverse correlation with MSI, we also observed an association between beta-catenin overexpression and prolonged survival from CRC.

Paper III

Aims

Activating mutations of the proto-oncogenes KRAS and BRAF are common in CRC, and indicate resistance to anti-EGFR drugs. Though most previous studies link KRAS and BRAF mutations to a poor prognosis, the prognostic relevance of these mutations has not yet been fully clarified. Further, KRAS protein functionality may vary, depending on the specific codon harboring a mutation. Here, we aimed to further investigate the correlations of KRAS codons 12 and 13, and BRAF mutations with clinicopathological factors and survival, overall and in subgroups according to gender and MSI status.

Summary of results

KRAS and BRAF mutational status was analyzed using pyrosequencing, and could be assessed in 525 and 524 cases, respectively. A total of 334 (63.7%) tumours were KRAS wild-type, and 191 (36.4%) were mutated. In specific, 156 (29.8%) cases harbored a mutation in KRAS codon 12, 34 (6.5%) in KRAS codon 13, and 1 (0.2%) in both KRAS codons 12 and 13. Further, 446 (85.1%) of the tumours were BRAF wild-type, 76 (14.5%) were BRAF V600E mutated and 2 (0.4%) were BRAF K601E mutated, with a total of 78 (14.9%) cases harboring a BRAF mutation. KRAS and BRAF mutations were mutually exclusive.

Looking at clinicopathological and molecular correlates, KRAS codon 13 mutation was associated with advanced M-stage ($p=0.018$), and inversely associated with p27 expression ($p=0.018$). KRAS codon 12 mutated tumours displayed a higher proportion of mucinous histology, compared to tumours that were either KRAS wild type ($p=0.032$) or codon 13 mutated ($p=0.024$). A significant correlation between KRAS wild-type and MSI ($p<0.001$) was further observed.

BRAF mutation was associated with older age ($p=0.017$), female sex ($p=0.031$), proximal tumour location ($p<0.001$), advanced T-stage ($p=0.001$), low differentiation grade ($p<0.001$), mucinous tumour type ($p=0.001$), MSI ($p<0.001$),

and expression of cyclin D1 (p=0.003), and further inversely associated with beta-catenin overexpression (p<0.001), p53 expression (p<0.001) and p27 expression (p<0.001).

Cox regression revealed that patients with KRAS wild-type and codon 12 mutated tumours had a similar prognosis, whereas KRAS codon 13 mutation was significantly associated with a reduced CSS in the full cohort (HR=1.94; 95% CI=1.18-3.19) and in women (HR=2.58; 95% CI=1.31-5.09), but not in men. Significance was not retained when adjusting for age, sex, TNM-stage, differentiation grade, vascular invasion and BRAF mutation. Moreover, BRAF mutation was significantly associated with an inferior CSS in men (HR=3.50; 95% CI=1.41-8.70) in adjusted, but not in unadjusted, analysis. No significant associations between BRAF mutation and survival were observed in the full cohort or in women.

We further investigated the impact of KRAS codons 12 and 13, and BRAF mutations on survival in subgroups according to MSI status, and in strata according to gender. Hereby, we observed that BRAF mutation was overall associated with a significantly shorter CSS in patients with MSS tumours (HR=2.36; 95% CI=1.44-3.86), however not independently. In men with MSS tumours, BRAF mutation was associated with an inferior CSS, both in unadjusted (HR=3.46; 95% CI=1.78-6.47) and adjusted analysis (HR=4.91; 95% CI=1.99-12.12). BRAF mutation was not prognostic in MSS tumours or in women, irrespective of MSI status.

Discussion

We here demonstrate that KRAS codon 13 mutation predicts a poor prognosis in female CRC, although not independently. KRAS codon 13 mutation was further associated with the presence of distant metastases at diagnosis, further supporting its association with a more aggressive tumour phenotype. Notably, the prognosis was similar for KRAS wild-type and codon 12 mutated tumours. Apparently, codons 12 and 13 mutations have different impact on KRAS protein functionality, and, hence, clinical outcome. Previous studies on the prognostic significance of KRAS mutations in CRC have reported conflicting results, although the majority link KRAS mutation (any mutation or in specific codons) to inferior survival [67,123,189,191-195]. Further, associations of KRAS codon 13 mutation with a poor prognosis [191,197] and advanced M-stage [191] have been reported previously, which validates our findings. The frequency and distribution of KRAS mutations were also in line with the expected [49], and revealed no sex differences.

Of note, KRAS mutation was inversely associated with MSI, which in turn is associated with a good prognosis and female sex [53,55]. Thus, a reasonable question is whether the poor survival observed in women with KRAS codon 13 mutated tumours merely represents the absence of MSI. KRAS mutation has further been associated with CIMP-low, CIN and aberrant MGMT methylation [51,60,61], and thus may indicate a distinct subgroup of CRC with a poor prognosis [51]. These studies did however not consider mutations in specific codons, and it would be of interest to investigate the associations between CIMP and specific KRAS mutations in future studies.

Moreover, we observed that BRAF mutation is an independent predictor of a poor prognosis in male, but not in female, CRC. Of note, this association was not significant in unadjusted analysis, which might be explained by a strong dependency between covariates, e.g. the particularly strong prognostic impact of BRAF mutation in lymph node positive disease in men.

Subgroup analysis according to MSI status revealed that BRAF mutation was not prognostic in patients with MSI tumours, neither in the full cohort nor in strata according to gender, and that the lack of prognostic value for BRAF mutation in women did not differ by MSI status. However, in male patients with MSS tumours, BRAF mutation was an independent predictor of a poor prognosis. These findings are in line with several previous studies reporting a particularly poor survival in patients with BRAF mutated, MSS, CIMP-high tumours [67,212,213,216,271]. Thus, it is becoming increasingly evident that it may not be the BRAF mutation per se that confers a poor prognosis. Rather, the effects of a BRAF mutation seem to differ depending on the genetic background in which it occurs, and perhaps, the oncogenic pathway that led to the development of the cancer. In this context, the results from our study suggest that sex may be another important determinator of the prognostic impact of BRAF mutation.

The clinicopathological and molecular correlations of BRAF mutation were in line with the expected [215,272]. Notably, BRAF mutation was significantly associated with MSI. Further, BRAF mutated tumours were, similarly to MSI tumours, associated with proximal tumour location, older age, female sex, advanced T-stage, low differentiation grade, mucinous histology, expression of cyclin D1, and inversely associated with beta-catenin overexpression, p53 expression and p27 expression.

BRAF mutations are frequently present in sporadic CRC with MSI, but almost never in HNPCC [273,274]. This indicates a connection between BRAF mutation and hypermethylation / CIMP, which has been confirmed in several studies [67,275,276].

KRAS and BRAF mutations were, as expected [207,277], mutually exclusive. Thus, while both factors seem to contribute equivalently to colorectal carcinogenesis, they represent different pathways to malignant progression.

Due to the EGFR-independent activation of KRAS and BRAF upon mutation, it is not surprising that these events indicate resistance to anti-EGFR drugs [125,208-210,278]. Thus, testing for KRAS and BRAF mutations has become clinical routine before administrating such drugs. Of note, the proportion of patients in our study that may have received anti-EGFR treatment upon relapse should be negligible. Therefore, it is not likely that our results regarding the prognostic impact of the investigative mutations have been confounded by effects of such treatment.

The sex-related differences in the prognostic value of KRAS codon 13 and BRAF mutations have, to our best knowledge, not been previously reported. These novel findings may indicate a hormonal involvement, and it would therefore be of great interest to further investigate the possible associations of KRAS and BRAF mutations with hormonal, anthropometric and lifestyle factors in CRC.

Taken together, the findings from this study further indicate that sex should be taken into consideration when evaluating biomarkers in CRC.

Paper IV

Aims

Upstream of KRAS and BRAF in the Ras/Raf/MEK/MAPK signaling cascade, the epidermal growth factor receptor (EGFR) plays an important role in regulating cell growth and differentiation. Commonly observed in CRC, EGFR protein expression, and EGFR gene copy number (GCN) alterations, have been linked to inferior survival from CRC and sensitivity to anti-EGFR drugs, respectively. In paper IV, our main ambition was to explore the impact of EGFR protein expression and GCN alterations on survival from CRC and on chemotherapy response. We further studied the interrelationship between EGFR protein expression and GCN alterations, and their correlations with clinical and investigative factors.

Summary of results

Expression of EGFR protein was evaluated with two different antibodies and systems, e.g. Zymed and Ventana (see Table 6). Membranous IHC staining intensity was recorded as 0 to 3 according to previous protocols [279]. Out of 553 cases evaluable with the Zymed antibody, 179 (33.6%) were denoted as having positive EGFR expression (1-3), and 114 (21.4%) as having high EGFR expression (2-3). Using the Ventana antibody, a total of 531 cases could be evaluated, of which 155 (29.2%) were EGFR positive and 103 (20.4%) displayed high EGFR expression.

EGFR gene amplification was assessed using brightfield double-in situ hybridization (BDISH), and was considered to be present when the EGFR / CEN7 ratio was ≥2 within 20 non-overlapping tumour cell nuclei. Ratios between 2 and 5 were considered as low-level amplification, and >5 as high-level amplification. Polysomy was defined as EGFR gene amplification as well as CEN7 signals with a ratio >2. GCN could be evaluated in 498 cases, of which 240 (48.2%) were non-amplified, 117 (23.5%) displayed low-level amplification, 41 (8.2%) high-level amplification and 100 (20.1%) polysomy.

Concordance between the two antibodies was good ($p<0.001$), with 95.5% of tumours being negative in both assays, and no tumour being negative with one antibody and scoring 3 with the other. There were further significant correlations between EGFR protein expression, assessed by both antibodies, and EGFR GCN variations denoted as non-amplified, amplified (any GCN) or polysomic, with the strongest correlation for the Zymed antibody ($p=0.008$). All p-values and hazard ratios below refer to analysis with the Zymed antibody, though similar results were obtained with the Ventana antibody.

EGFR protein expression, dichotomized as high (2-3) vs. low (0-1), was significantly associated with proximal tumour location ($p=0.004$), advanced T-stage ($p<0.001$), N-stage ($p=0.002$), M-stage ($p=0.003$), low differentiation grade ($p<0.001$), vascular invasion ($p=0.003$), BRAF mutation ($p<0.001$), and inversely associated with beta-catenin overexpression ($p=0.015$) and p27 expression ($p<0.001$).

Furthermore, EGFR gene amplification and polysomy correlated significantly with distal tumour location ($p=0.005$), MSS ($p<0.001$), p53 expression ($p<0.001$), and BRAF wild-type tumours, and an increased EGFR GCN was associated with distant metastasis ($p=0.037$).

Survival analysis showed that high EGFR protein expression was significantly associated with a reduced CSS in the full cohort (HR=2.04; 95% CI 1.50-2.78), in stage I-II disease (HR=2.30; 95% CI 1.16-4.53) and in stage III-IV disease (HR=1.54; 95% CI 1.08-2.19). After adjustment for established prognostic factors, significance was retained in the full cohort (HR=1.60; 95% CI 1.11-2.31) and in stage III-IV disease (HR=1.73; 95% CI 1.14-2.64), but not in stage I-II disease. Increased EGFR GCN was significantly associated with a reduced survival in the full cohort (HR=1.65; 95% CI 1.20-2.26) and in stage III-IV disease (HR=1.56; 95% CI 1.07-2.64), but neither in stage I-II disease, nor in adjusted analysis. Subgroup analysis revealed no sex-related differences in the prognostic significance of EGFR expression or GCN alterations.

We further examined the impact of EGFR expression and GCN alterations on chemotherapy response in curatively treated patients with stage III-IV disease. Here, we observed that in patients receiving adjuvant oxaliplatin (FLOX/XELOX), both high EGFR protein expression and GCN alterations were associated with a significantly reduced CSS (HR=7.46; 95% CI 1.19-46.61 and HR=6.16; 95% CI 1.03-36.69, respectively). A borderline significant interaction for oxaliplatin treatment (vs. no treatment or FLV/Xeloda) was observed for high EGFR protein expression ($p=0.057$), whereas no significant interaction was observed for EGFR GCN alterations.

Discussion

In this study, we have demonstrated that both EGFR protein expression and GCN alterations are associated with a poor prognosis in CRC. EGFR protein expression, in particular, was an independent predictor of a poor prognosis in the full cohort, and in stage III-IV disease. In addition, EGFR protein expression correlated significantly with adverse clinicopathological factors, such as advanced T-, N- and M-stage, low differentiation grade and vascular invasion. Several previous studies have come to similar conclusions, linking EGFR expression to advanced disease stage [218,222], adverse characteristics such as tumour budding [223], and inferior survival [220,223,224,226,280]. Altogether, our findings add further weight to the feasibility of EGFR as a predictor of poor prognosis in CRC.

Interestingly, EGFR expression has been associated with a poor response to radiation therapy (RT) of rectal cancer [224,281,282], although the mechanisms remain unclear [283]. Nevertheless, this may further contribute to the poor prognosis associated with high EGFR expression.

The association of EGFR expression with advanced T-stage is not surprising, considering the important role of EGFR in cell proliferation and cell cycle progression [284,285]. In this context, the inverse association between expression of EGFR and the cell cycle inhibitor p27 was also expected [220]. Nonetheless, we found no significant associations between EGFR alterations and cyclin D1 expression.

An increased EGFR GCN has been suggested to predict responsiveness to anti-EGFR drugs [228,230,235,286], but has to our best knowledge not previously been reported as a prognostic marker in CRC.

Even though the correlation between EGFR protein expression and EGFR GCN alterations was statistically significant, there was a substantial discrepancy in their interrelationship. For example, only 55.1% of tumours being negative for EGFR protein expression were also non-amplified. Similar discrepancies have been previously reported in CRC [230,231,233]. Moreover, there was a discordance in the correlations of EGFR expression and GCN alterations in that EGFR expression was associated with proximal tumour location and BRAF mutation, whereas GCN alterations correlated with distal tumour location and BRAF wild-type tumours. There is no obvious explanation for this discrepancy, but, of note, elevated EGFR protein expression may occur due to other mechanisms than gene amplification; e.g. activating mutations, increasing EGFR transcription or translation, decreased protein destruction, and overexpression of receptor ligands [287].

As it has been proposed that EGFR mutation precedes gene amplification, both steps resulting in EGFR protein expression, in a sequential tumour progression model [288], it would also be of interest to analyze EGFR mutation status of the herein investigated tumours in future studies.

Moreover, the lack of standardization of detection methods and interpretation of results may also explain the divergent results in the literature [236], with reported EGFR expression rates from 16 to 97% in CRC [218]. Intratumoural heterogeneity of EGFR expression may also be an interfering factor [280].

Of note, an increased EGFR GCN can be caused by either gene amplification or polysomy of chromosome 7, and both seem to be relevant when predicting response to anti-EGFR drugs [235]. Our findings further support that both EGFR gene amplification and chromosome 7 polysomy have a similar prognostic impact, and similar clinicopathological correlations. Thus, the dichotomized cutoff applied in the survival analyses should be justified.

Interestingly, our results also indicate that EGFR expression and GCN alterations are associated with an impaired response to oxaliplatin. This finding is, to our best knowledge novel, and may be of clinical importance. It should however be pointed out that the subgroups available for analysis were rather small, thus limiting statistical power. Therefore, these results need additional validation, preferably in randomized treatment trials.

Another observation of potential clinical relevance is that the prognostic value of EGFR alterations was strongest in stage III-IV disease, also including palliatively treated patients. According to current treatment protocols, anti-EGFR treatment is only given in late palliative situations or as neoadjuvant therapy, and in combination with irinotecan- or oxaliplatin based chemotherapy. Given that EGFR expression indicates responsiveness to anti-EGFR treatment, and further, resistance to oxaliplatin, it can be assumed that patients with KRAS wild-type and EGFR overexpressing / amplified tumours benefit from combination therapy with an anti-EGFR agent and irinotecan.

Conclusions

In brief, the results in this thesis can be summarized as follows:

- Nuclear cyclin D1 expression is less frequently expressed in male, compared with female, CRC.
- Nuclear cyclin D1 expression is associated with a favorable prognosis, however not independently, in stage III-IV male CRC.
- MSI is associated with distinctive clinicopathological features, cyclin D1 expression, and independently predicts a good prognosis in stage III-IV CRC.
- Beta-catenin overexpression correlates independently with a prolonged survival from stage III-IV CRC, and is associated with MSS tumours.
- KRAS codon 13 mutation predicts a poor prognosis in female CRC, but not independently of established prognostic factors.
- KRAS and BRAF mutations are mutually exclusive, and correlate with MSS and MSI, respectively.
- BRAF mutation is independently associated with a reduced survival in male patients with MSS CRC.
- EGFR protein expression is an independent factor of poor prognosis in stage III-IV CRC.
- An increased EGFR GCN is associated with a reduced survival from CRC, but not independent of established prognostic factors.
- EGFR protein expression correlates significantly with EGFR GCN alterations, although a substantial proportion of EGFR expressing tumours display a normal GCN, and vice versa.
- EGFR alterations are significantly associated with a reduced survival in curatively treated patients with stage III-IV disease receiving adjuvant oxaliplatin.

Future perspectives

Despite substantial advancements in the management of CRC during the recent decades, the disease still harvests more than 600000 lives globally each year. Moreover, it is becoming increasingly evident that CRC is a heterogenous disease, which affects survival and adjuvant treatment response beyond what can be predicted by conventional clinicopathological factors. Hence, an essential challenge in the ongoing efforts to combat CRC is to find new biomarkers to better predict the prognosis and stratify patients for adequate adjuvant treatment.

To date, KRAS mutation is the only biomarker implemented in clinical practice, as a predictor of resistance to anti-EGFR treatment in the palliative setting. Hopefully, more biomarkers will prove to be clinically relevant in the near future.

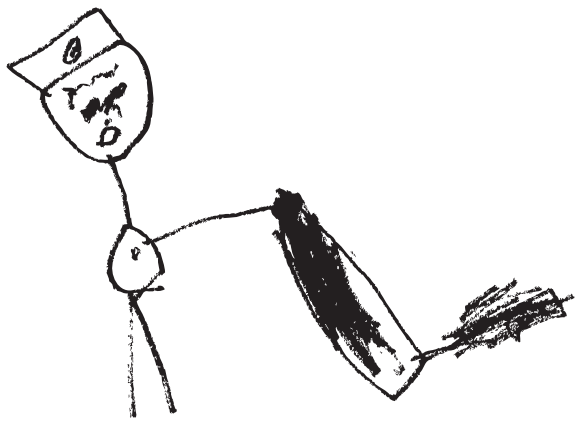
In this thesis, we have observed several sex-related differences in the distribution, clinicopathological associations, and impact on survival of the investigated biomarkers. These findings demonstrate that sex should always be considered when evaluating putative biomarkers in CRC, and that hormonal factors have considerable influence on the pathogenesis and progression of the disease.

To better understand the observed association of cyclin D1 with a favorable prognosis in male CRC, it would be of interest to study potential sex differences in the prevalence, clinicopathological correlates and prognostic significance of cyclin D1 polymorphisms in this cohort. As previously mentioned, the cyclin D1 G870A polymorphism has been suggested as a low penetrant risk factor for CRC [255].

Another relevant avenue of research would be to assess CIMP status of the tumours in this cohort, and to study the associations of CIMP with BRAF and specific KRAS mutations. KRAS mutation, without consideration of the specific codon, has previously been linked to CIMP-low [61], but, to our best knowledge, the associations of specific KRAS mutations with CIMP have not yet been reported.

The observed discrepancy between EGFR protein expression and GCN alterations indicates that EGFR signaling may be increased by a number of mechanisms. Since EGFR mutation has been demonstrated to precede gene amplification sequentially [288], it would be of interest to analyze EGFR mutation status of the tumours in this cohort, with particular reference to its associations with EGFR expression, alterations and clinical outcome.

In paper IV, we also observed that EGFR alterations indicate resistance to oxaliplatin. This finding is of potential clinical importance, and merits further investigation, preferably in randomized clinical trials.



Populärvetenskaplig sammanfattning

Tjock- och ändtarmscancer är en av de vanligaste cancerformerna, med mer än 1 miljon nya fall varje år globalt. Bara i Sverige insjuknar mer än 6000 individer årligen. Merparten av de drabbade är över 65 år och tjocktarmscancer, men inte ändtarmscancer, är något vanligare hos män. Både livsstilsfaktorer, såsom en västerländsk diet, och ärftlighet i samspel antas ligga bakom utvecklingen av tjock- och ändtarmscancer. Sambanden är dock långt ifrån klarlagda. Endast i ca 5% av fallen är sjukdomen direkt ärftlig.

Tack vare framsteg inom diagnostik och behandling har dödligheten minskat de senaste decennierna, men alltjämt dör över 600000 individer av sjukdomen varje år. Den enda botande behandlingen är kirurgi, som ibland åtföljs av tilläggsbehandling med cytostatika (cellgifter) för att minska risken för återfall.

För att kunna bedöma prognosen, och ännu viktigare avgöra vem som ska få tilläggsbehandling med cytostatika, delar man in sjukdomen i kliniska stadier från I-IV beroende på hur djupt tumören växer in i tarmväggen samt förekomsten av spridning till lymfkörtlar och andra organ. Prognosen varierar från mycket god när tumören är begränsad till tarmen, till dålig vid spridd sjukdom.

Det är dock uppenbart att den konventionella stadieindelningen är otillräcklig för att förutspå sjukdomsförloppet hos enskilda patienter. I vissa fall växer och sprider sig tumören snabbt, även om den upptäckts i ett tidigt stadium, medan förloppet i andra fall är mer beskedligt och långsamt, trots att sjukdomen hunnit sprida sig till andra organ vid diagnos. Det står också klart att utvecklingen från normal tarmslemhinna till cancer kan ske på olika sätt, och att de genetiska förändringar som cellerna samlar på sig under denna process påverkar tumörens aggressivitet och beteende. Att identifiera biomarkörer, vanligen proteiner som uttrycks av tumörerna, för att bättre kunna identifiera patienter med en mer aggressiv sjukdom och på så vis ge rätt behandling till rätt patient, är alltså av yttersta vikt.

I denna avhandling har vi studerat förekomsten av ett antal olika potentiella biomarkörer i tumörer från drygt 500 patienter med tjock- och ändtarmscancer, samtliga deltagare i den befolkningsbaserade studien Malmö Kost Cancer. Den gemensamma nämnaren för de biomarkörer som studerats är att alla är proteiner med en central roll i olika aspekter av omvandlingen från normal cell till cancer.

I första delarbetet analyserades förekomsten av proteinet cyclin D1 i tumörernas cellkärnor. Cyclin D1 reglerar celldelning och ses ofta överuttryckt i olika

cancerformer. Metoden vi använde, immunohistokemi, går kortfattat ut på att målsökande antikroppar binder till proteinet, vilket ger en färgreaktion som sedan kan studeras i mikroskop. Vi kunde konstatera att tumörer från män hade ett starkare uttryck av cyclin D1 jämfört med tumörer från kvinnor. Vidare kunde vi se att uttryck av cyclin D1 var kopplat till en bättre överlevnad hos män, men inte hos kvinnor.

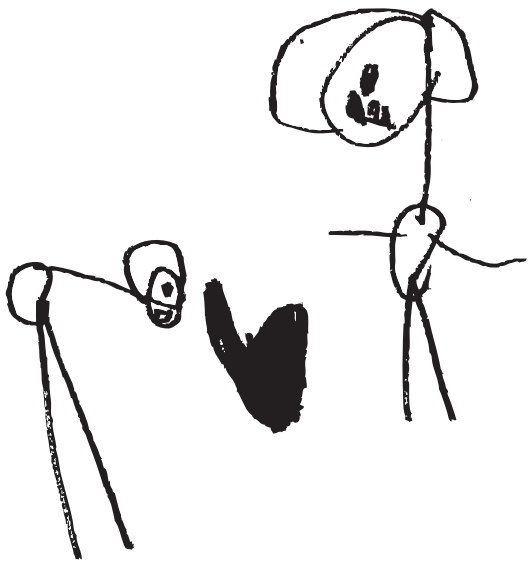
I nästa delarbete undersökte vi med hjälp av immunohistokemi förekomsten av mikrosatellitinstabilitet och förändringar av beta-cateninuttryck i tumörerna. Mikrosatellitinstabilitet innebär att tumörcellerna har nedsatt förmåga att korrigera de fel som uppstår i samband med DNA-replikation och celldelning, vilket resulterar i frekventa felaktigheter i DNA-sekvensen. Beta-catenin i sin tur är ett protein som dels reglerar bindningen mellan celler och dels förmedlar signaler från cellytan in mot cellkärnan. Vi kunde här konstatera att mikrosatellitinstabilitet var kopplat till en god prognos. Vidare var mikrosatellitinstabila tumörer, som ofta var stora, lokaliserade till höger sida av tjocktarmen, samt slembildande, överrepresenterade hos äldre kvinnor. Även ett ökat uttryck av beta-catenin var kopplat till en bättre överlevnad, om än inte lika tydligt som för mikrosatellitinstabila tumörer.

I det tredje delarbetet utforskade vi mutationer i två gener som heter KRAS och BRAF. Båda kodar för proteiner som har en viktig funktion i att vidarebefordra signaler från receptorer på cellytan in till cellkärnan och mutationer i dessa gener, som leder till ohämmad cellsignalering och därmed tumörcellstillväxt, är vanliga vid tjock- och ändtarmscancer. Med hjälp av pyrosekvensering, som är en metod för att bestämma DNA-sekvenser, kunde vi identifiera vilka tumörer som var muterade och vilka specifika mutationer ifråga det rörde sig om. Här kunde vi observera att en viss typ av KRAS-mutationer, men inte alla, var kopplade till en dålig prognos, framför allt hos kvinnor. Mutationer i BRAF var förknippade med en dålig prognos hos män vars tumörer inte uppvisade mikrosatellitinstabilitet.

I det sista delarbetet intresserade vi oss för EGFR (epidermal growth factor receptor), en viktig tillväxtfaktorreceptor som sitter på cellytan och känner av yttre kemiska signaler. Vid stimulering fortplantar EGFR signalerna in till cellkärnan, och KRAS och BRAF fungerar som viktiga ”strömbrytare” i denna process. Vi analyserade dels uttrycket av proteinet EGFR med hjälp av immunohistokemi, och dels studerade vi antalet kopior av EGFR-genen med hjälp av s.k. in situ hybridisering, en metod som är principiellt snarlik immunohistokemi, men där man istället använder målsökande s.k. prober mot en viss DNA-sekvens eller gen. Resultaten visade att högt uttryck av proteinet EGFR var kopplat till en dålig prognos. Detta gällde även ett ökat antal EGFR-genkopior, om än sambandet inte var lika starkt. Vi såg däremot ett klart samband mellan EGFR proteinuttryck och ett ökat genkopieantal, även om många tumörer som uttryckte EGFR proteinet hade ett normalt antal genkopior och vice versa. Slutligen tittade vi på hur EGFR

proteinuttryck och genkopieantal påverkar överlevnaden hos patienter som behandlats med cytostatika. Det visade sig att såväl högt EGFR proteinuttryck som ökat genkopieantal var kopplat till sämre svar på behandling med en viss typ av cytostatika (oxaliplatin).

Sammanfattningsvis kan vi med detta avhandlingsarbete konstatera att tumörer i tjock- och ändtarm i många avseenden skiljer sig åt mellan kvinnor och män. Detta gäller inte bara risken att drabbas av sjukdomen utan även hur prognosen avspeglas i uttrycket av olika biomarkörer. Därutöver har vi identifierat nya biomarkörer som kan förutspå effekt av cytostatikabehandling, oberoende av kön. Det behövs dock ytterligare studier innan dessa biomarkörer är redo att tas i kliniskt bruk.



Acknowledgements

Jag vill rikta ett särskilt tack till:

Karin Jirström, min huvudhandledare. Det har varit otroligt inspirerande och roligt att vara din doktorand! Tack för ditt outtömliga engagemang och stöd. Du är verkligen en fantastisk person!

Jakob Eberhard, för utmärkt bihandledarskap. Tack för entusiasm och perspektiv. Det har varit ett nöje!

Jonas Manjer, som varit min bihandledare och dessutom introducerade mig för Karin!

Doktorandgruppen: Karolina Boman, Jenny Brändstedt, Jacob Elebro, Richard Fristedt, Charlotta Hedner, Liv Jonsson och Anna Larsson. Vad roligt vi har haft det!

Ex-doktorander Björn Nodin och Alexander Gaber, för all hjälp och för att ni är de ni är!

Nooreldin Zendehrokh, för hjälp med pyrosekvensering.

Medförfattare Kajsa Ericson Lindquist, Magnus Sundström och Göran Elmberger för analyshjälp och expertis.

Diana Karpman och mina kursare på Forskarskolan, för lärorika och inspirerande veckor.

Bengt Jeppsson, för att du ordnat så att jag fått den forskningstid jag behövt.

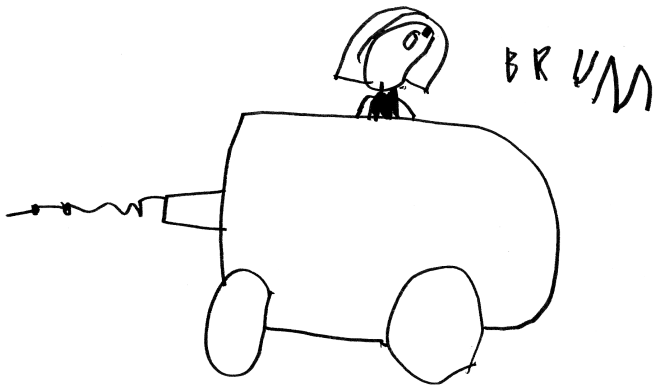
Mina kollegor på kirurgiska kliniken. Bättre gäng får man leta efter!

Claes och Ingrid, mina föräldrar. Tack för villkorslös kärlek och oändligt stöd!

Chatarina, min älskade fru. Du är mitt allt.

Aron och Bror, finaste killarna i världen!

Lillasyster, för att du väntade lite med att komma så jag hann skriva klart!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