HER3 EXPRESSION AND PROGNOSIS IN COLON AND RECTAL CANCER

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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My hand is weary with writing,
My sharp quill is not steady,
My slender-beaked pen jets forth
A black draught of shining dark-blue ink.

A stream of wisdom of blessèd God
Springs from my fair-brown shapely hand:
On the page it squirts its draught
Of ink of the green-skinned holly.

My little dripping pen travels
Across the plain of shining books,
Without ceasing for the wealth of the great—
Whence my hand is weary with writing.

11th-century poem in honour of St Colum (nästan colon) Cille about Book of Kells

CONTENTS

ABSTRACT

In Sweden, about 6000 patients get a colorectal cancer diagnosis annually. Advances in management of both colon and rectal cancer have reduced mortality even though the incidences are increasing. To improve outcome further, it is important to identify prognostic and predictive factors for personalized, optimal surgery and to guide adjuvant treatment decisions. By selecting the right therapy combined with selection of the right patients, one can obtain individualization. Potential drug targets prevalent in primary tumors and metastases are of interest as well. HER3 is a transmembranous, epidermal growth factor receptor that is over expressed in colon and rectal cancer. It affects cellular proliferation, differentiation and migration in embryogenesis and in oncogenesis through activation of intracellular signal pathways.

This thesis investigates HER expression and prognosis in colon and rectal cancer, as well as in correlated lymph node and liver metastases. HER3´s association to a combination of biomarkers, MMR expression and HLA-A*02 genotype, is examined related to prognosis.

Two cohorts of patients have been available for our investigation. Immunohistochemical (IHC) detection of HER3 and MMR expression was performed in a group of Swedish patients with primary colon and rectal cancer of stage II and III. The patients derived from a randomized Nordic trial aiming to evaluate additive effect of adjuvant chemotherapy to surgery. HER3 expression was also detected by IHC in a different group of patients, resected for both colorectal cancer (CRC) and corresponding liver metastases. FISH analysis was done to examine if gene amplification occurred associated to HER3 expression and HLA-A*02 genotype was assessed by PCR.

In the initial study of patients with primary CRC of stage II and III, a high HER3 expression was seen in 70% of tumors and in 75% of lymph node metastases. Tumor and lymph node metastases correlated according to HER3 expression. HER3 did turn out to be an independent prognostic factor and high expression was associated to decreased survival. FISH analysis of CRC tumors did not show gene amplification with respect to HER3 expression.

A high expression of HER3 was seen in about 80% of primary CRC as well as in corresponding lymph node and liver metastases. There was a correlation between HER3 expression in primary tumor and metastases.

In the third study, high HER3 expression in colon cancer was associated to distal colon location and low-grade tumor. In distal colon cancer, high HER3 expression was of negative prognostic value according to disease free survival (DFS).

The results in our last study indicate that a combined analysis of HER3, MMR expression and HLA-A*02 genotype can have a prognostic value and can, to some extent, predict response to adjuvant 5-fluorouracil (5-FU) based chemotherapy in subgroups of primary colon cancer.

A single molecular biomarker or a combination of markers may play a relevant prognostic and predictive role though CRC is a complex and heterogeneous disease. Subtyping of CRC based on molecular, clinical and morphological features includes biomarkers and is difficult however, necessary when planning optimal treatment for each individual patient.

Key words: Colorectal cancer, colon cancer, HER3, IHC, lymph node metastases, liver metastases, prognosis, tumor location, biomarker, differentiation, MMR, HLA-A*02, adjuvant chemotherapy

LIST OF SCIENTIFIC PAPERS

The thesis is based on the following articles, referred to in the text by Roman numerals.

I **HER3 expression in patients with primary colorectal cancer and corresponding lymph node metastases related to clinical outcome.**

Lédel F, Hallström M, Ragnhammar P, Öhrling K, Edler D.
European Journal of Cancer, 2014 February; 50(3):656-62

II **HER3 expression in primary colorectal cancer including corresponding metastases in lymph node and liver.**

Lédel F, Stenstedt K, Hallström M, Ragnhammar P, Edler D.
Acta Oncologica, 2015 April; 54(4):480-6

III **HER3 expression is correlated to distally located and low-grade colon cancer.**

Lédel F, Stenstedt K, Hallström M, Ragnhammar P, Edler D.
Accepted in Acta Oncologica, December, 2015

IV **A combined analysis of HER3, MMR and HLA-A*02 in colon cancer stage II and III.**

Lédel F, Villabona L, Masucci G, Hallström M, Ragnhammar P, Edler D.
Submitted manuscript, December, 2015

UNRELATED PAPER

The expression of CYP2W1 in colorectal primary tumors, corresponding lymph node metastases and liver metastases.

Stenstedt K, Hallstrom M, Lédel F, Ragnhammar P, Ingelman-Sundberg M, Johansson I, Edler D. *Acta Oncologica,* 2014 July; 53(7):885-91

THESIS AT A GLANCE

	Hypothesis	Patients and methods	Results	Conclusion
I	The expression of HER3 in primary tumor of CRC is elevated and correlates to expression in lymph node metastases. HER3 is associated to survival and prognosis.	CRC, stage II and III, n=236, IHC, FISH	70% of tumors had high HER3 expression and 75% of lymph node metastases. High HER3 expression was a negative prognostic factor. Correlation of HER3 expression between tumor and metastases was observed. No gene amplification existed.	High HER3 was detected in 70% of tumors, in 75% of lymph node metastases and correlated mutually. High HER3 expression was prognostic and predicted a worse outcome.
II	The expression of HER3 in CRC, in lymph node and liver metastases is elevated and expression in tumor and metastases correlates.	Resected CRC + liver metastases, n=107, IHC	80% of primary tumors had high HER3 expression, 81% in lymph node and 82% in liver metastases. Correlation in HER3 expression between tumor, lymph node and liver metastases was observed.	High HER3 expression was seen in about 80% of CRC and corresponding metastases. Correlation existed between HER3 expression in tumor and metastases.
III	The expression of HER3 in primary colon cancer is elevated. HER3 is associated to prognosis and tumor location.	Colon cancer, stage II and III, n=521, IHC	High HER3 was expressed in 67% of colon tumors. High HER3 expression was associated with distal colon and low-grade tumor. In patients with distal tumor, high HER3 expression correlated to shorter DFS.	In this enlarged, refined study, high HER3 expression in colon cancer was associated to distal colon and low-grade tumor. High HER3 expression was of negative prognostic value in distal tumors.
IV	A combined analysis of biomarkers HER3, MMR and HLA-A*02 sharpens prognostic value in colon cancer.	Colon cancer, stage II and III, n=493, IHC, PCR	Correlation between high HER3 expression and proficient MMR existed. When adjuvant therapy was given to high HER3/pMMR patients, a tendency to prolong DFS was seen compared to only surgery. In females with stage III, receiving surgery alone, the combinations high HER3/HLA-A*02 and proficient MMR/HLA-A*02 had worse outcomes compared to not having HLA-A*02.	A combined analysis of HER3, MMR and HLA-A*02 held prognostic value and a tendency to prediction was observed regarding adjuvant chemotherapy in subgroups of patients.

LIST OF ABBREVIATIONS

5-FU	5-Fluorouracil
APC	Adenomatous polyposis coli
BRAF	Proto-oncogene B-Raf
CEA	Carcinoembryonic antigen
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CRC	Colorectal cancer
CT	Computed tomography
CTL	Cytotoxic T-lymphocyte
d/pMMR	Deficient/proficient mismatch repair
DFS	Disease free survival
FAP	Familial adenomatous polyposis
FFPE	Formalin-fixed paraffin embedded
FISH	Fluorescence in situ hybridization
HER3	Human epidermal growth factor receptor 3
HLA	Human leucocyte antigen
HNPCC	Hereditary non polyposis colorectal cancer
IHC	Immunohistochemistry
KRAS	Kirsten rat sarcoma viral oncogene
mCRC	Metastasized colorectal cancer
MHC	Major histocompatability complex
miRNA	Micro ribo nucleic acid
MLH-1	MutL homolog 1
MRI	Magnetic resonance imaging
MSH-2	MutS homolog 2
MSI	Microsatellite instability
OS	Overall survival
PET	Positron emission tomography
PCR	Polymerase chain reaction
TS	Thymidylate synthase
WNT	Wingless-type

INTRODUCTION

Cancer in general

The problem; suffering and dying people!

Cancer is a leading cause of death worldwide, accounting for 8,2 million deaths of which 694 000 constitutes of colon and rectal cancer (2012) [1].

Causes, why cancer?

Cancer arises from one single cell. It is a multistage process when a normal cell transforms into a tumor cell. These changes are the result of the interaction between one individual´s genetic factors and three categories of external agents;

Physical (e.g. radiation) *Chemical* (e.g. tobacco, food) *Biological* (e.g. intestinal flora)

Ageing is also fundamental for the development of cancer. The incidence of a specific cancer rises with age and that is due to a build-up of risks. Risk accumulation interacts with the cellular repair mechanisms that become less effective as a person grows older.

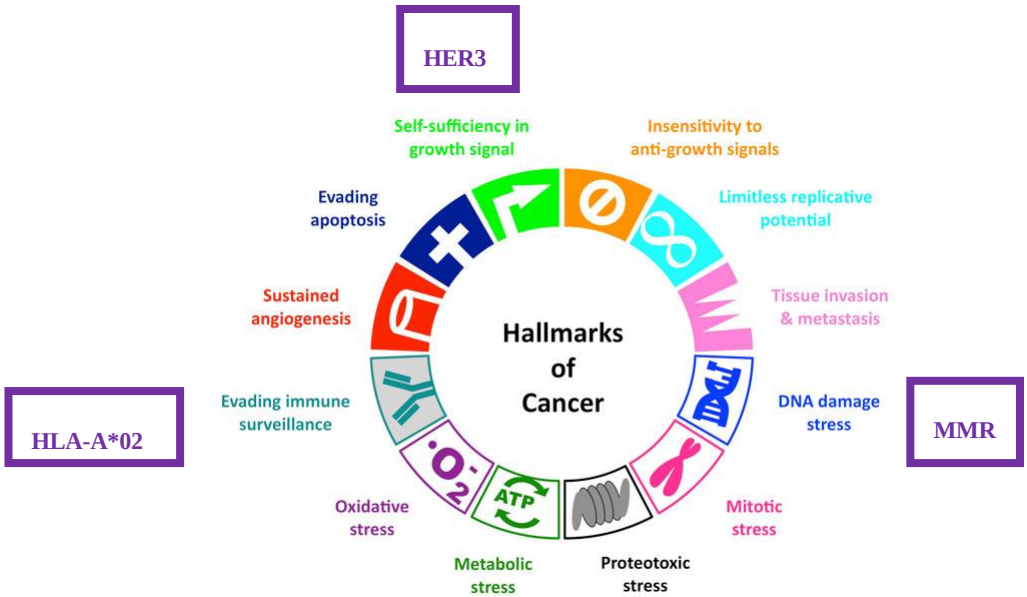


Figure 1. *Ref;* Hanahan and Weinberg 2011

Risk, "big five"

A large proportion, 30%, of cancer deaths could be prevented. Tobacco, alcohol, unhealthy diet, physical inactivity and some chronic viral infections (hepatitis B and C, HIV and HPV) are the "BIG FIVE" of the cancer safari.

Reduce cancer risk, HER3?

Through cancer prevention (the dream is to be able to use HER3 here), early detection (the dream is to also use HER3 here) and management of patients (we might have reasons to use HER3 here) the cancer risk can decrease. Maybe, I can contribute with this work to reduce the cancer burden.

Colon and rectal cancer

Colon and rectal cancer are diseases of welfare where the highest incidences are found in Europe, USA and Oceania. In Sweden, 6162 colorectal cancer (CRC) cases were diagnosed in 2013. CRC is the third most common cancer disease [2]. The age standardized mortality has decreased and overall survival has increased during the last 30 years [3]. The age specific incidence of CRC shows the same characteristic pattern for colon and rectal cancer, common in the elderly and unusual among the young [4]. Since both incidence and survival of CRC have increased during the last decades, the number of patients treated for the disease has grown. As for several other tumor forms, there is no single triggering factor known for CRC. Etiological factors can be carcinogens in feces and mutagens in Western food. The geographical variation of CRC incidences is scattered and can change within a generation when moving from a low risk to a high risk area. This might be explained by lifestyle or environmental factors like e.g. red meat, poor intake of fruit and vegetables, physical inactivity, obesity, tobacco and alcohol. Though the evidence for etiological factors are not so strong, a reduction of up to 30% of CRC have been estimated and could be prevented by change of food and lifestyle [5]. HLA genotypes differ in expression around the world in an evolutionary way and may also have an impact in CRC [6]. Other general features of CRC are that men have a slightly higher risk of getting adenomas as well as CRC and preferably distal colon and rectal tumors. Women tend to have an overweight of proximal tumors, especially at a higher age [3]. Diabetes, widespread, chronic ulcerative colitis and Crohn´s disease augment the risk of CRC [7, 5]. Age is the single most important risk factor followed by heredity for CRC in the clinic.

Colon cancer

The trend of incidence for colon cancer in Sweden is increasing over decades but in the last 5 years, it is actually declining. Another trend is that fewer deaths of colon cancer are seen in

the last 30 years (Figure 2). The 5-year survival rates for all stages of colon cancer were 65% for females and 62% for males in 2009 to 2013 [4].

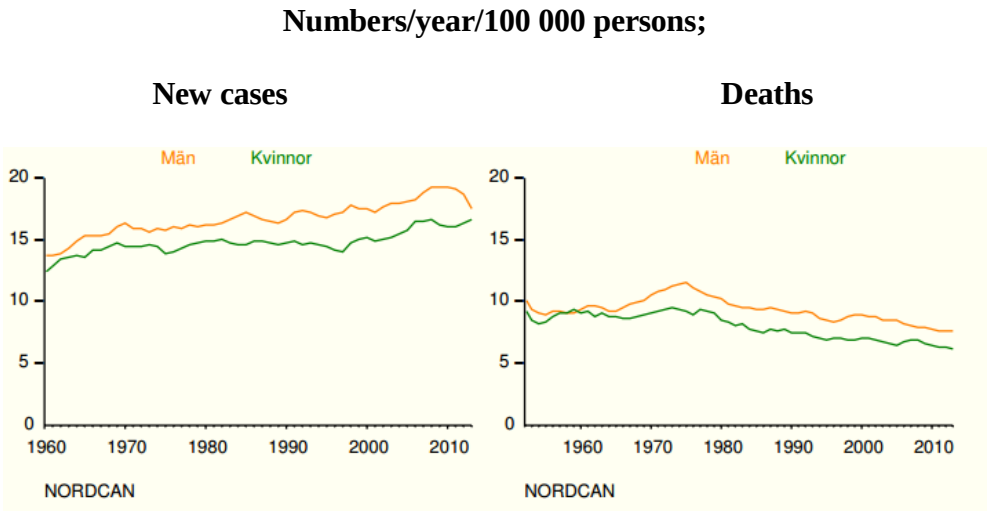


Figure 2. Colon cancer
Adapted from Nordcan

Rectal cancer

The trend of incidence for rectal cancer in Sweden is slightly increasing but flatter compared to colon cancer over decades. Like colon cancer, the trend is that fewer deaths of rectal cancer are seen in the last 30 years (Figure 3). The 5-year survival rate for all stages of rectal cancer is 66% for females and 62% for males from 2009 to 2013 [4].

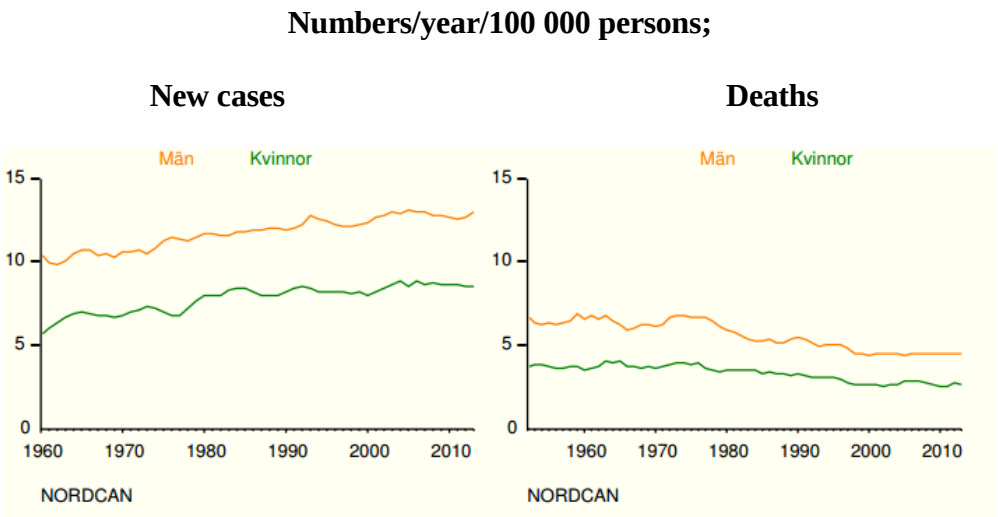


Figure 3. Rectal cancer
Adapted from Nordcan

Symptoms

The majority of patients with CRC are asymptomatic. The symptoms can differ according to tumor location in proximal, distal or rectal cancer. Changed habits of defecation, blood or mucus in the stools, urgency, pain to defecate, abdominal pain (constant or intermittent), weight loss, anemia and abdominal swelling are all common symptoms. Large bowel obstruction which constitutes of 20% of the onset of all CRC, bleeding or tumor associated perforation are late symptoms [8].

Prevention

Colorectal tumors are suitable for screening in a biological point of view but optimal methods of investigation, which are easy to use, free of risk to patients, with a high sensitivity and specificity, are not fulfilled. As of today in literature, screening can probably result in increased survival in subgroups but might not affect the total CRC mortality [5, 8]. Screening gains in colorectal cancer with fecal occult blood detection and colonoscopy for patients at the age of 60 are investigated in Sweden in the SCREESCO trial [9].

Investigation

To start the investigation with a colonoscopy and rectoscopy to get a biopsy and acquitting from synchronous tumors are gold standard in CRC. Computed tomography (CT) colography can be performed instead of colonoscopy but without the important biopsy opportunity. Further, CT is done of the thorax and abdomen for a radiological, preoperative staging of the tumor and to detect metastases. A rectal digital exam should be done in the clinical examination. For rectal cancer, magnetic resonance imaging (MRI) is always done because of the clear anatomical vision of the pelvic space [10]. Positron emission tomography (PET-CT) is used to map a locally advanced, primary tumor or to evaluate if the metastatic situation is curative or palliative. MRI or ultrasound with contrast of the liver can be of complement in the preoperative investigation or in the follow up surveillance and radiological controls. Trans rectally performed ultrasound can be of complement to MRI. The tumor marker carcinoembryonic antigen (CEA) assessed from a blood sample, is used in clinical practice as a diagnostic tool as well as in the follow up of CRC to detect recurrences [11].

Multidisciplinary team conference

During the last decade, multidisciplinary team (MDT) conferences have been established in Sweden. These conferences are structured meetings with colorectal surgeons, oncologists, radiologists, pathologists and specialized nurses and when needed, liver and thoracic surgeons present. Each patient´s colorectal cancer case is individually discussed at least at two occasions, pre- and post-operative and again if a recurrence occurs. In a complicated case, the patient is discussed more frequent. Decisions concerning pre- and post-operative staging, treatment and follow up are made at the MDT. The aim of the conference is to tailor the optimal surgical and oncological treatment for each individual patient. Improved survival is a hard endpoint that is reached since implementation of MDT conferences [12]. Further systematic review of the MDT´s is needed. A panel of different biomarkers is becoming standard in MDT settings of other solid tumors e.g. breast cancer and lung cancer, which are of help when making each patients very best decision [13, 14]. In CRC, microsatellite instability (MSI) status and *KRAS* mutation of the tumor are sometimes complemented at the MDT to determine if adjuvant chemotherapy or epidermal growth factor receptor inhibitor e.g. cetuximab are going to have effect. HER3 expression or HLA-A*02 genotyping are not yet used in the clinic.

Treatment

Surgery

Surgery is the primary treatment of CRC. Time between diagnosis and surgery should be minimized and no longer than 6 weeks. Curative resection of CRC is the strongest associated factor of survival of the patient [8]. The surgical techniques in CRC have changed stepwise with e.g. the total mesorectal excision (TME) in rectal cancer, “high thigh” ligation of colonic vessels as proximal to the aorta as possible in all bowel resections and taking the complementary mesentery *en bloc*, harvesting as many lymph nodes as possible [15, 16, 17, 18]. These improvements have all together prolonged survival. Morbidity and mortality related to surgery have decreased due to a more accurate pre-operative investigation, modern anesthetic and surgical techniques. Open, laparoscopic or robotic approaches of CRC surgery are used. Principles of operation of CRC are resection of the tumor affected bowel segment with an adequate marginal. Bowel resection is done *en bloc* with corresponding mesentery, containing lymph vessels and regional lymph nodes. Resectable, distal metastases (e.g. suspected malignant nodes of the para aorta, obturatorius foramen, groin or liv-

er/lung metastases) are taken away either at the operation of the primary tumor, before or after, regarding scenario. The entire abdominal cavity should always be examined for visible or palpable metastases [8]. Lymph node extirpation (regional and resectable, metastatic distal) is necessary for the TNM-staging of the tumor but can also have a therapeutic relevance.

The different resections of the colon and rectum for tumors are; right and left hemicolectomy (extended or not), transeversectomy, total and subtotal colectomy (synchronous tumors, FAP or HNPCC), sigmoidectomy and rectal resection or amputation. Acute colon resections for colon cancer are done in 20% of all cases and should, if possible, be avoided [19]. An obstructing tumor is the most common cause but also perforation or bleeding can be reasons for acute surgery. Pre-operative, TNM-staging is strongly recommended even if the setting is acute or semi acute. Acute colon cancer surgery is more demanding then elective and is often performed out of regular hours and without colorectal competence. For patients having acute resections of colon cancer, the prognosis is negatively affected [19]. The procedure can be more extensive due to the condition of bowels compared to elective surgery. Stoma of some kind is more common and often recommended in the acute setting [20]. Common complications of bowel surgery are leakage of the anastomosis, infections (wound, abdominal abscesses, pneumonia, urinary tract) and rarely occurring today, bleeding.

Radiation

Radiation has a direct tumor cell killing effect but can also sensitize cancer cells to be found by the immune response or recruit the immune response itself. Radiation is given to rectal cancer patients to reduce the risk of local recurrence [21]. If radiation has an effect, the tumor visibly shrinks and/or fibrosis transformation of the tumor is seen on MRI or in the pathology report. Preoperative given radiation according to standard (5x5 Gray) of rectal cancer has decreased the local recurrence rates [8]. The border between resectable and unresectable tumor is not razor sharp. The MRI can present three different tumors; favourable “good” group, intermediate “bad” group and advanced “ugly” group [22]. Many studies have been conducted on how and to whom to give radiation (optimal dose, administrative pattern, tumor type) and if it should be combined with chemotherapy [23, 24]. The category of “good” does not need radiation. The second category, “bad”, constitutes the largest group of patients and the risk of local recurrence is higher than the expected morbidity of radiation itself so standard radiation is recommended. In the category of “ugly”, radiation and neo adjuvant chemothera-

py is definitely needed to get a radical resection, to prevent local recurrence and in some cases to obtain shrinkage and convert an inoperable tumor to an operable case [25, 26]. Radiation can cause a complete response in some patients, which means that the tumor is no longer radiologically or pathologically detected [27]. MRI staging is the superior modality for choosing the right patients that need radiation but the tumor tissue biopsy also matters. Local recurrence risk has decreased because of better surgery and radiation but survival has not significantly improved yet. RAPIDO is an ongoing, randomized, multicenter study where patients of high risk of recurrence are included [28]. The hypothesis is that chemotherapy combined with radiation in rectal cancer can add in systemic effect and increase tumor control locally, which might result in increased survival in advanced tumors. Radiation of colon cancer is not usually performed because of the non-fixed position of bowel tumor and simultaneously risk of damage to the small bowels, which are sensitive to radiation. However, radiation of long term and high dose can be considered in locally advanced colon cancer cases without metastases combined with chemotherapy [29].

Adjuvant chemotherapy

The hard endpoint of systemic adjuvant chemotherapy in resected CRC patients is to eradicate the micro metastases that might exist [30]. While the pre-operative staging has improved, surgery and pathology have as well, thus resulting in stage migration. This means that the stage specific recurrence risk and stage survival have changed but not in total. This is known as the Will-Rogers phenomena [31]. 5-Fluorouracil (5-FU) based chemotherapy is standard adjuvant treatment for stage III colon cancer [32, 33]. Adjuvant 5-FU and e.g. calcium folinate (potentiates 5-FU) started within 8 weeks of surgery and given for 6 months to patients <76, can reduce risk of recurrence with 30-40%. The 5-year survival increases about 10% [24, 32, 34]. Capecitabine (fluoropyrimidine), is an orally administrated prodrug of 5-FU and is an equivalent to intravenous 5-FU and calcium folinate [35]. The enzyme thymidylate synthase (TS) and DNA synthesis are inhibited by 5-FU. Leucovorin, calcium folinate and other similar agents stabilizes the binding of 5-FU metabolite, fluorodeoxyuridine monophosphate to nucleotide-binding site of TS, forming a ternary complex [33, 36].

Supplementary treatment to 5-FU is given to patients with high-risk tumors that can tolerate it. Oxaliplatin is an inhibitor of DNA replication, inhibiting topoisomerase that blocks DNA repair and is a down-regulator of TS [37]. Neuropathy is the main side effect of oxaliplatin

that can be permanent. Patients older than 70 years old probably have more side effects than gains of oxaliplatin but can tolerate 5-FU though biological age should be considered [38]. Both cetuximab and irinotecan are used in the metastatic CRC setting. For a simple overview see Table 1 below.

Adjuvant treatment	**Additional adjuvans**	**Metastatic treatment**
5-FU/calcium folinate or leucovorin	Oxaliplatin	5-FU/calcium folinate or leucovorin
Capecitabine		Capecitabine
		Oxaliplatin
		Irinotecan
		Cetuximab
		Bevacizumab
		Panitumumab

Table 1. Overview of CRC and oncological treatment.

More documented research exists in the colon cancer and adjuvant chemotherapy field compared to rectal cancer. Studies are now conducted for rectal cancer but the evidence base is still not completely solid. In general, colon and rectal cancer patients of stage II are not given adjuvant chemotherapy and colon cancer of stage III do get chemotherapy. Selected rectal cancer patients of stage III get adjuvant chemotherapy. The stage specific risk of recurrence or death, not regarding treatment, can be seen in Table 2.

Stage	**Risk of recurrence or death in 3-5 years**
II (20-30%)	20-40%
III (30-40%)	40-60%
IV (20%)	~85%

Table 2. Risk and stage in CRC.

Stage II patients with colon cancer at risk with e.g. high-grade tumor, tumor vessel growth, T4 or few harvested lymph nodes, tumor perforation and relative low age are all parameters that often render in an adjuvant chemotherapy decision. If tumor perforation exists, the risk for recurrence is high which motivates adjuvant chemotherapy. Colon and rectal cancer patients of stage III get adjuvant chemotherapy with a believed beneficial effect of 10-20% [32]. Oral capecitabine or intravenous 5-FU+calcium folinate with or without oxaliplatin do have a proven effect but is to a high extent dependent on risk factors, patient and tumor characteristics [39]. It is clinically relevant to even more specifically identify stage II and III patients that we know would or would not benefit from adjuvant chemotherapy. If guidelines of CRC

and adjuvant chemotherapy from the Swedish National Board of Health and Welfare are followed, recurrence or premature deaths are estimated to be prevented in 2-4/100 patients [3, 8]. The use of biological agents like cetuximab, panitumumab, bevacizumab and others have an effect in metastatic CRC (mCRC) but not yet a proven effect as neo adjuvant or adjuvant treatment. The ways to administrate chemotherapy are orally or intravenously.

Colon cancer and chemotherapy

5-FU has been given as adjuvant chemotherapy in colon cancer for many decades. The additive effect of 5-FU to surgery is favorable in about 7% in colon cancer of stage II and III [32, 33]. A study from the nineties [36] and following studies still hold strong and patients with colon cancer stage III receive adjuvant chemotherapy today. The side effects are tolerable and only a few late side effects are seen of chemotherapy [33]. If oxaliplatin is given additionally to 5-FU/calcium folinate or capecitabine to patients with colon cancer stage III, the relative risk of recurrence decreases another 20%. In stage II it is still not completely clear if the gains of oxaliplatin are as large as in stage III [40, 41].

Rectal cancer and chemotherapy

Increased survival of the combination of radiation and neo adjuvant or adjuvant chemotherapy has been hard to prove in rectal cancer [24, 42]. Results from large scale colon cancer trials have been extrapolated to give adjuvant chemotherapy to rectal cancer patients of stage III and patients at risk in stage II. In non-radiated and radiated patients with tumors presenting a high grade of risk factors, oxaliplatin can be given additionally to adjuvant chemotherapy. Since TME surgery and radiation have been combined, the loco regional problem in rectal cancer is almost eliminated [43]. Neo adjuvant radiochemotherapy in rectal cancer patients is shown to improve local control in the advanced, “ugly”, group of tumors, but does not improve survival [21, 44]. The gain of adjuvant chemotherapy after neo adjuvant chemotherapy is unknown. If a tumor is down staged by radiation and chemotherapy, the gain of adjuvant chemotherapy is probably low [43, 45].

CRC, metastases and chemotherapy

CRC with limited liver and/or lung metastases can be operated with a curative intention more than once. If these patients are selected in a proper way, a 5-year survival of 45-50% is observed compared to all stage IV patients where the 5-year survival is much lower [46].

Neo adjuvant, adjuvant or peri-operative chemotherapy have been given in these cases after a MDT decision [47, 48]. The resection of CRC and liver metastases can be done synchronous or at two occasions depending on the level of difficulty. The EPOC study has shown that a sandwich model of peri-operative administration of chemotherapy, FOLFOX (5-FU+calcium folinate+oxaliplatin) and cetuximab prolongs DFS [47]. CRC and lung metastases are operated at two occasions.

Biological therapies

As an additional treatment to chemotherapy, four antibodies are used which are tyrosine kinase inhibitors (TKI); bevacizumab (VEGF inhibitor), cetuximab and panitumumab (EGFR inhibitors) and regorafenib (multitarget inhibitor). These new drugs have a well documented but restricted effect and are expensive. Cetuximab and panitumumab can be given without the combination of cytostatics in mCRC and give a remission of 10% in unselected patients. *RAS* wildtype (*KRAS* and *NRAS*) in the tumor is a requirement for the EGFR inhibitors to work and raises remission with 20-30% [49, 50]. The two drugs are similar in the third line of mCRC in a randomized study [51]. Irinotecan can be combined with e.g. cetuximab and render patients an improved survival [52]. Other prognostic markers like HER3 expression can interact with TKI´s.

Heredity

Familial syndromes are estimated to cause 25% of CRC but only 5% have an identified genetic defect [53]. Risk of hereditary cancer is when an individual has inherited a mutated gene that makes the person more prone to developing cancer. There are families where several members will develop colon or rectal cancer. In such families, cancer occurs more often than would have been expected by chance yet is not detectable hereditary. Little is known about the causes of cancer in these families [53]. Epigenetic interactions might occur between genes or gene clusters and the environment. This type of moderately increased cancer risk is named "familial colon and rectal cancer." Genetic testing for hereditary CRC can be done from a blood sample.

Hereditary nonpolyposis colorectal cancer (HNPCC)

HNPCC, also known as Lynch syndrome, is the most common form of hereditary colon cancer, accounting for ~3% of all colorectal cancer each year [54]. It should be suspected

when CRC is diagnosed at a low age or heredity is present. Although not each and everyone with HNPCC will develop colorectal cancer, the risk is greatly increased compared with the general population. The CRC risk is approximately 80% over a lifetime. In HNPCC patients, colonoscopy with polypectomi when needed, each 1-2 years should be performed, starting at 20-25 years of age. This procedure lowers the risk of CRC and extends expected survival [55]. The risk of developing other cancers in e.g. the endometrium, ovaries or prostate is increased in HNPCC. The genes that have been associated with HNPCC are inherited germline mutations in DNA mismatch repair (MMR) genes (*MLH-1, MSH-2, MSH-6* and *PMS-2*) (Figure 10) [56]. The consecutive effect is widespread microsatellite instability (MSI).

Familial adenomatous polyposis (FAP)

FAP is a rare hereditary condition in which the patient has hundreds to thousands of polyps in the colon and rectum. FAP accounts for approximately ~1% of all colorectal cancer each year [57]. The polyps are adenomas and not cancer. However, they have the potential to develop into cancer. The polyps begin to occur in teenage years and malignification is complicated to monitor. Colonoscopy is recommended annually from 12 years of age until total colectomy [58]. The majority of tumors develop in the distal colon and rectum. If the colon of a person with FAP is not removed, eventually CRC will be developed. FAP patients can also develop polyps in other gastrointestinal (GI) locations and there is an increased risk of GI cancers, desmoids, thyroid cancers and medulloblastoma. Non-cancerous features of FAP include soft tissue tumors like osteomas, skin cysts, dental abnormalities and congenital hypertrophia of the retinal pigment [59]. The gene associated with FAP is germline mutated *APC* that is an early and crucial step in the adenoma carcinoma sequence. Patients with FAP have a mutation in the *APC* gene, which can be inherited (Figure 7) [60].

MYH-gene associated polyposis (MAP) is another inherited, rare form which develops later in life than FAP. People with MAP and FAP have very similar polyp development and the only way to distinguish between the syndromes is with genetic testing [60].

Other forms of hereditary colon cancers are; depending on chromosomal location or single nucleotide polymorphism (SNP´s). Juvenile Polyposis, Peutz-Jegher's syndrome and PTEN hamartomas have a greater risk for CRC [53].

Embryology and Tumor location

Epidermal growth factors mediate their effect on epithelial cells in the colon and rectum through binding to naturally located epidermal growth factor receptors at the basolateral site of the cell membrane. The epidermal growth factor receptors play a major role in embryonic development, morphogenesis and maintenance of the colonic and rectal epithelium as well as in oncogenesis [61]. Survival, migration and mitosis of stem cells and pre-differentiated cells resulting in mature epithelial cells are regulated by the epidermal growth factor receptor (Figure 4).

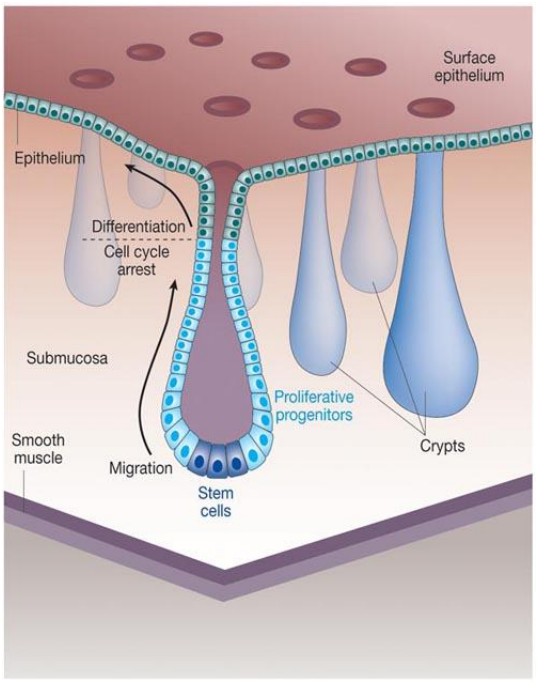


Figure 4. Proliferation, migration and differentiation from stem cells to epithelial cells in the submucosal bowel crypts
Adapted from Nature Reviews Cancer

In CRC, this order of proliferation and differentiation of cells is changed and epidermal growth factor receptors are over expressed in an abnormal, distributional pattern in the complete cell membrane [62]. In all epithelial cell types, epidermal growth factor receptors exist which illustrate their modulatory functions on various differentiated cells of the colon crypt [62]. In a fetus, epidermal growth factor receptors increase in number during gestation and at the same time, ligand affinity decreases but in cancer this autonomal control is lost [63]. Epidermal growth factor receptors EGFR, HER2, HER3 and HER4 are important targets for clinical applications in cancer detection and therapy because they regulate proliferation, invasion, angiogenesis and metastases of cancer cells [63]. The location of tumors related to expression of epidermal growth factor receptors have been studied (Figure 5). EGFR, HER2 as well as HER3 are over expressed throughout the colon but at a higher extent in distal colon and rectal tumors compared to proximal tumors [64, 65].

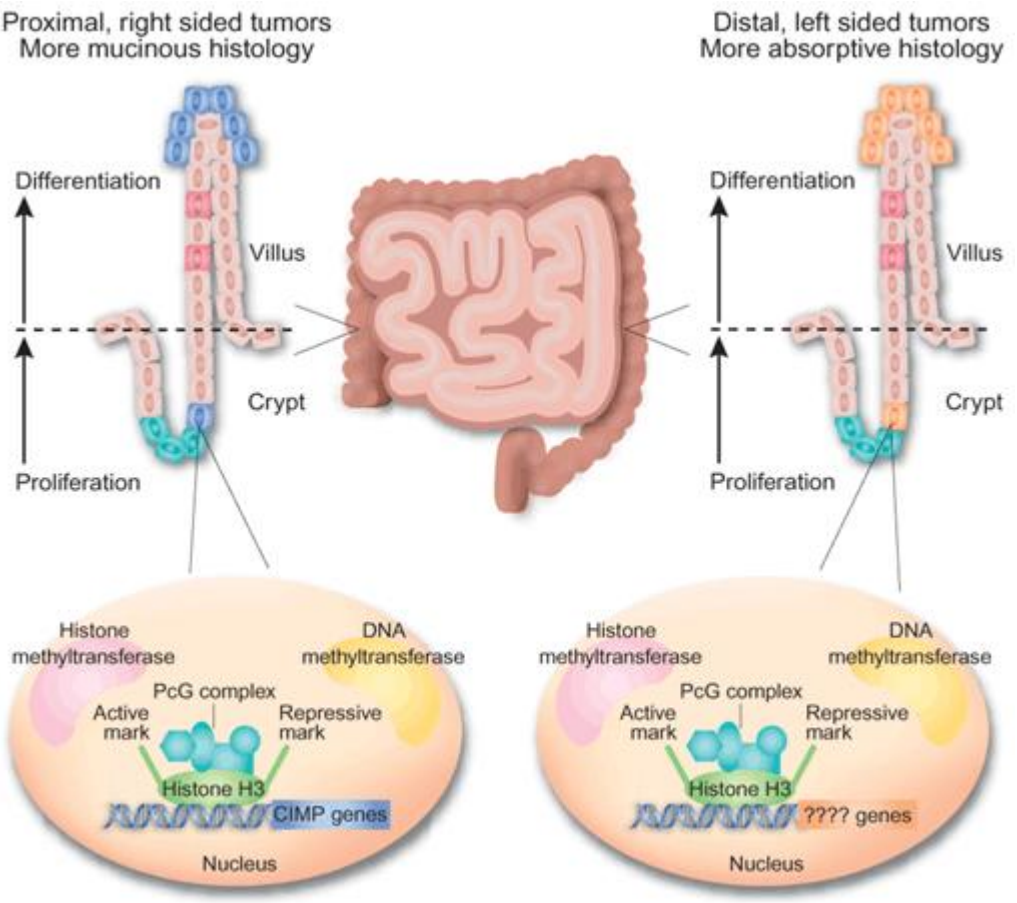


Figure 5. Tumor location related to phenotype and genetic background.
Adapted from Nature Reviews Cancer

One distinctive embryonic feature is the rudiment of midgut and hindgut resulting in proximal and distal colon. The caecum, ascending and 2/3 of the transverse colon derive from the midgut while the colonic segment including splenic flexure, left colon, sigmoid and rectum, derive from the hindgut. The dual blood supply of the colon reflects the different embryonic origins. Regarding innervation of the colon and rectum, it´s divided, where proximal colon is supplied by the vagus nerve, while the distal colon and rectum are maintained from the S2, S3 and S4 [66]. The distinction between the colon and rectum is anatomical but surgical and radiotherapeutic management differs. Tumor location is potential with prognostic and predictive implications [67, 68].

Staging

Diagnostic information in the pathology report is based on the macro as well as the microscopic tissue analysis of a CRC tumor and is decisive for endoscopic or surgical removal and for neo adjuvant or adjuvant treatment [69]. It additionally gives feedback to the MDT on the radiological tumor, node, metastases (TNM) staging, quality of surgery and results of oncological treatment. The pathologist is recommended to use the latest WHO Classification of Tumors of Digestive System 2010, as of date, the 7th edition of UICC, TNM Classification of Malignant Tumors [70].

T is how deep in the bowel wall the tumor is infiltrating. N gives information on the number of regional lymph node metastases. M is if distant metastases exist. Stages 0 to IV are used (Table 3).

AJCC stage	TNM stage	TNM stage criteria for colorectal cancer
Stage 0	Tis N0 M0	Tis: Tumor confined to mucosa; cancer-in-situ
Stage I	T1 N0 M0	T1: Tumor invades submucosa
Stage I	T2 N0 M0	T2: Tumor invades muscularis propria
Stage II-A	T3 N0 M0	T3: Tumor invades subserosa or beyond (without other organs involved)
Stage II-B	T4 N0 M0	T4: Tumor invades adjacent organs or perforates the visceral peritoneum
Stage III-A	T1-2 N1 M0	N1: Metastasis to 1 to 3 regional lymph nodes. T1 or T2.
Stage III-B	T3-4 N1 M0	N1: Metastasis to 1 to 3 regional lymph nodes. T3 or T4.
Stage III-C	any T, N2 M0	N2: Metastasis to 4 or more regional lymph nodes. Any T.
Stage IV	any T, any N, M1	M1: Distant metastases present. Any T, any N.

Table 3. TNM and stage.

As an addition to TNM, certain validated risk factors are used to predict recurrence and metastases (Table 4) [70].

Risk factors of recurrence and metastases in CRC
T4, N1-2, M
Number of analyzed lymph nodes <12
High grade tumor
Vascular invasion
Lymphatic invasion
Perineural tumor growth
Tumor deposits
Tumor budding
Infiltrating tumor border
Tumor perforation

Table 4. Pathological risk factors of CRC.

Pathology

According to the simplified paradigm of a CRC tumor, cancer arises from an adenoma estimated to take many years to malignify (Figure 6) [71].

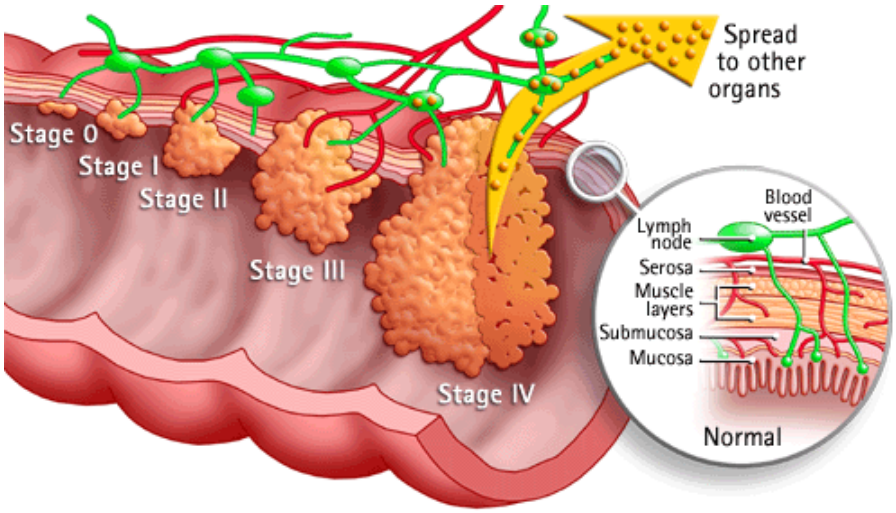


Figure 6. Growth of tumor in the bowel wall and stages of CRC.
Adapted from National Cancer Institute

Surgical resection is the most effective treatment of CRC. Histopathological examination of the tumor specimen according to the TNM system can predict prognosis. The depth of tumor infiltration in T3 tumors is related to prognosis and is independent of stage. T4 tumors can break through the serous surface and have a worse prognosis compared to being spread to adjacent organs [72]. Dukes staging system (A-D) is today replaced by the 0-IV staging sys-

tem. The number of analyzed lymph nodes is of importance for correct staging and an indicator of surgical and pathology quality and the ruling ground for adjuvant chemotherapy decision [72]. If more than 12 negative regional lymph nodes are found, this is predictive of stage I or II [73]. Low-grade cancer is defined to have ≥50% glandular formations and are well to moderately differentiated while high-grade cancer has ≤50% glandular formations and are low differentiated [72]. Tumor budding is a single cell up to a small cluster of undifferentiated, stemlike cells adjacent to the tumor. It is suggested as the first event of migration of cancer cells and is a negative prognostic factor [74]. Tumor deposits are larger clusters of tumor cells which are a kind of micro metastases. These are often seen close to the tumor as well [75]. Two types of tumor borders are identified, pushing or infiltrating where the latter is associated to recurrence risk and metastases [74].

The surgeons are recommended to adequately mark important anatomical structures and send the specimen fresh or in formalin to the pathology department. A minimizing of autolysis should be pursued. A tumor is after resection, conserved through formalin fixation and paraffin embedded (FFPE). Sliced FFPE tumor sections (3-5µm) are fixed on glass slides and multiple analyses can then be done, including IHC. From tumor tissue, one can extract DNA from fresh but also from fresh-frozen or formalin-fixed paraffin embedded tumor. Quality of DNA and DNA degradation are depending on the e.g. dilution of formalin solution, time to fixation and fixation time. DNA can be used for various types of molecular genetic analyses. Tissue micro array techniques are micro biopsies from the same or different paraffin embedded tumors that are analyzed at the same time with immunohistochemistry (IHC) or fluorescence in situ hybridization (FISH). This is a time and tissue sparing method which enables multiple biomarker analyses in a number of tumors. For clinical practice in CRC, we are not there yet. Today only MSI-testing and *KRAS* (PCR) are reliable, validated biomarkers. Biobanks of CRC tumors are now established for clinical trials and for individual hospitals. This is going to be of great importance for future research possibilities regarding prognostic refinement and for identifying predictive factors of treatment. All biobanking need informed patient consent, ethical committee approval and a report to the National Board of Health and Welfare Registry of Biobanks.

Inflammation and Immune response

Long lasting inflammatory activity, e.g. ulcerative colitis, predisposes for CRC and in a lifetime, 18% of malignancies occur [76]. A combination of colitis, having risk factors of CRC and heredity can have a large effect on incidence and prognosis. Aberrant activities of

JAK/STAT signaling pathways have been implicated in the development and spread of various cancer entities, among them CRC [77]. An equilibrium exists between immune response and CRC where the JAK/STAT pathway has an impact. The JAK/STAT complex is present in e.g. lymphocytes and is activated by interferons, interleukins, cytokines and growth factors. STAT1 and 3 expression and activity constitute as independent favorable prognostic markers for CRC, where STAT protects the individual from cancer [77].

MSI tumors also present this balance between the immune system fighting the tumor while the tumor is trying to evade immune response [78]. Genetic MMR deficiency results in MSI tumors and represents a specific phenotype in CRC. Epigenetic silencing of DNA mismatch repair and hypoxia have been shown to affect inflammatory bowel disease-associated CRC [79]. Elevated microsatellite alterations at selected tetranucleotide repeats called EMAST is a commonly presented form of MSI that is initiated by inflammation and modulates CRC progression [80].

The HLA-A*02 genotype might have an impact on prognosis since it is associated to ovarian tumors and colon tumors of advanced stages and to a worse prognosis [81, (Villabona et al. "Analysis of immune-related prognostic markers in colon cancer in patients randomized to surgery or surgery and adjuvant cytostatic treatment", submitted 2015)]. One hypothesis behind this is that the HLA-A*02 genotype impairs immune response in CRC.

The HER complex is present in all epithelial cells in a normal state and in an aberrant pattern in CRC. Very little is known of HER3´s over expression in CRC and its relationship to immune response. What is evident though is that when inhibiting antibody treatment like cetuximab is given, acquired resistance occurs and HER3 might have an impact [82].

Information on HER3 expression in sporadic colon tumors and how it interacts with MMR and HLA-A*02 genotype is sparse. Different HER complex members can be targets for CTL´s in cancer cells. HER complex expression has been reported to associate to MHC class I. Over expression of HER complex might impair CTL mediated recognition of HLA-A*02 restricted tumor antigens [83, 84].

Metastases in CRC

The definition of metastases is the presence of tumor growth, away from the tumor, in distant lymph nodes or in other organs, documented by radiology, pathology or clinical examination. The survival is poor in CRC of stage IV (Table 2) [85]. The most common locals of CRC metastases are in the liver, the lung, para aortal lymph nodes and other distal lymph nodes

[86]. Among CRC patients, 15-20% have liver metastases at diagnosis, another 20% metastasize metachronously [87]. Even if CRC is spread to the liver, it is potentially curable with surgery in 20% of all patients and it has markedly increased survival [87]. When investigating tumor biology of synchronous and metachronous colorectal liver metastases and molecular marker expression, it is concluded that most genetic aberrations in the primary tumor are maintained in the CRC liver metastases [88]. Synchronicity might imply a more aggressive disease though biological differences between primaries of synchronous and metachronous groups have been difficult to prove [89]. *KRAS* mutational status, which is important when considering anti EGFR therapy, is maintained in primary CRC and in corresponding metastases [90]. In metastatic CRC treated with EGFR inhibitors while having high HER3 expression, patients had a worse outcome compared with a low HER3 expressing tumor [91]. This implicates again that HER3 might interact with the response and with acquired resistance to anti-EGFR therapy in cancer [92]. A gene expression signature of the CRC metastases has been suggested and a high expression of these genes in the primary tumor correlated with worse outcome [93].

Genetic and epigenetic development in CRC

Adenoma Carcinoma Sequence

Adenomas are the most important lesions for carcinoma development as well as the subgroup of hyperplastic polyps. It is a continuous progression from normal epithelium to aberrant crypt foci, to adenoma, to cancer and finally, to metastases. Approximately 10% of adenomas progress to CRC and it takes about 10-15 years [94]. With rising age, about 50% of the population has one or more adenomas in the colon. The size of the adenoma is associated to high-grade dysplasia [95]. The adenoma carcinoma sequence proposes that specific mutations appear chronologically when a normal cell transforms to a carcinoma with a potential of metastatic behavior (Figure 7).

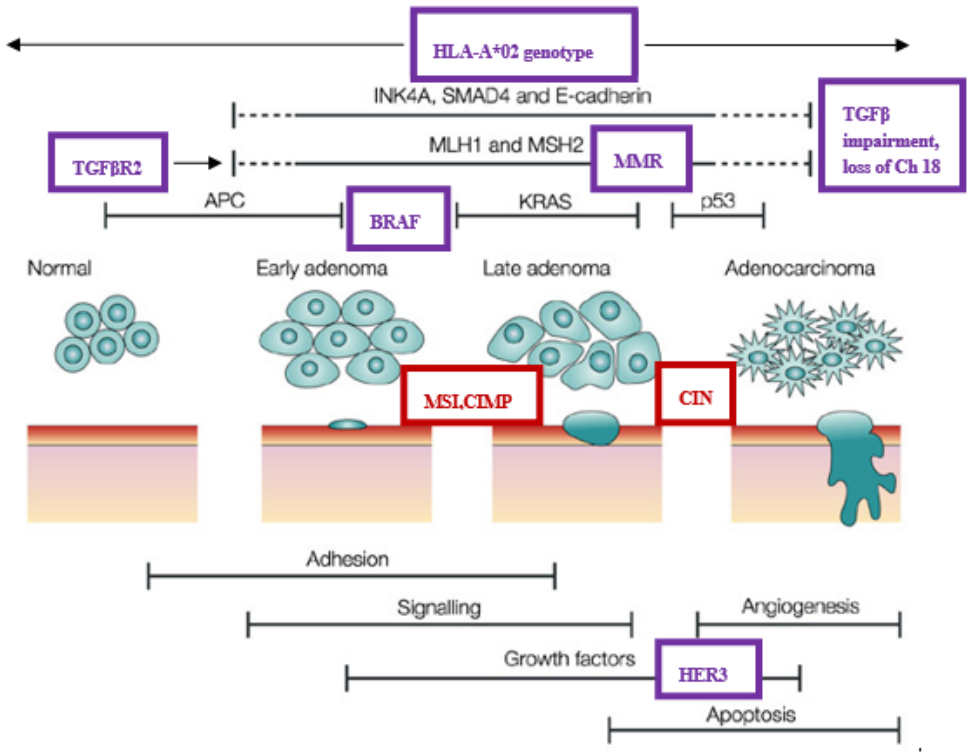


Figure 7. Markers of oncogenetic events can be seen in purple boxes and phenotypes in red boxes.

Modified from Nature Reviews Cancer

These mutations can affect genes and pathways that regulate differentiation and growth [96]. Mutated proto-oncogenes cause "gain of function", which means uninhibited proliferation even in the absence of growth signals. Tumor suppressor genes handle "loss of function" in

the cell cycle and when these genes are silenced or mutated, the normal regulation and balance between proliferation and apoptosis is disturbed.

The *APC* gene mutation, found in ~80% of CRC, is considered the initial event when a cell transforms into an adenoma [94]. The *APC* mutation is called the "gate keeper" of CRC. When the WNT pathway is active it increases proliferation in a cell and this is an early event in the adenoma carcinoma sequence. When *APC* is mutated, the WNT pathway is on nonstop and the cell proliferates [97]. A similar mechanism as for WNT is observed for mutated oncogene *KRAS*, seen in ~40% of CRC. *KRAS* is active between early and late adenoma transformation. *KRAS* is located at chromosome 12 and associated with growth of the adenoma through regulation of multiple functions in different pathways [98]. Mutated *BRAF* is another proto-oncogene that affects the sequence in early malignant evolution and interacts with the MAPK pathway. *BRAF* mutation is less frequent (<10%) than *KRAS* in CRC [99]. The cell proliferation rate will increase with *KRAS* and *BRAF* mutations. *SMAD4* and *TGFβR2* mutations also contribute in the malignifying process in CRC. *TGFβR2* is considered a driver of MSI [98]. Late events in the sequence are mutated tumor suppressor gene, *TP53,* "the guardian of the genome" (>50% of CRC) as well as impairment of the whole TGFβ pathway and loss of chromosome 18q (includes SMAD 2 and 4) [98].

An alternative pathway from Fearon and Vogelsteins in CRC is observed for hyperplastic polyps where mutated *BRAF* starts the process and epigenetic events like promotor methylation and gene silencing keep it going [100]. MSI tumors arise through a methylated or mutated *MLH-1* gene or others. Hyperplastic polyps leading to CpG island methylation phenotype (CIMP) and MSI tumors are more frequently seen in proximal colon [101, 102].

The MSI, CIMP and CIN phenotypes

Parallel to the adenoma carcinoma sequence, there are three groups that are both independent and mutually overlapping (Figure 7).

For solid tumors, chromosomal aberrations like aneuploidy or polyploidy are of importance. In CRC, about 85% of the tumors follow the adenoma carcinoma sequence and present numerical chromosomal alterations called chromosomal instability (CIN) [98]. In CIN tumors, chromosomal composition drives recurrent gains and losses that affect chromosomes in a non-random manner. The cause of CIN is not known, but it is suggested that alterations in the mechanism apparatus when chromosomes segregate in mitosis might be a

cause. CIN tumors are distally located, of low-grade, more often have malignant nodes and metastases and do not have peri-tumoral lymphocytic infiltrate when compared to MSI tumors. Associated mutated genes in CIN tumors are *APC, KRAS* and *PI3K* (Figure 7) [98].

Tumors in which microsatellite mutations are demonstrated are called microsatellite instable (MSI) and exist in 15% in sporadic CRC but also in HNPCC [102]. The MSI phenotype (described in page 42) has defects in the mismatch machinery where errors that are introduced during replication, are left unrepaired [102]. At least four genes are involved in dMMR in humans *MLH-1, MSH-2, PMS-2* and *MSH-6* (Figure 10) [56]. Deficient MMR genes can cause MSI due to either mutations or hyper methylation of promotors that silence MMR genes. The order of succession is that dMMR in sporadic cancer occurs after mutations of *APC* and *KRAS* followed by a cascade of oncogenetic mutations (Figure 7) [103].

The methylation described above is an epigenetic chemical modification of DNA folding that leads to gene expression changes called CIMP [103]. Sporadic biallelic silencing of e.g. the *MLH-1* gene is a form of CIMP. Many genes have promoters embedded in clusters of cytosine-guanosine residues, CpG islands. The CIMP phenotype resembles the MSI phenotype but is *BRAF* mutated, absent of MLH-1 and PMS-2 proteins through methylated silencing of the *MLH-1* gene and is diploid. The cause of CIMP is also unknown [103]. Both MSI and CIMP are consequences of a defect MMR system but through different mechanisms. When using IHC, which measures protein expression, both causes are covered. The CIN and MSI phenotype initially were considered mutually exclusive but now are found to partly overlap. The CIMP and MSI phenotype are also overlapping to a large extent [102, 103]

Biomarkers in CRC

The definition of a biomarker is a biological factor that can be of various sorts; peptide, receptor, gene, allele or combinations that are used for risk prediction, as a diagnostic tool, for prognosis or prediction of treatment outcome [104].

The growing knowledge about molecular mechanisms of cancer in general has augmented expectations that compounds and abberations associated to oncogenesis can be used as biomarkers. In a typical CRC, about 80 genes are mutated whence only a small fraction is found in the majority of patients and most mutated genes are present at low frequencies. Therefore, a wider perspective then genetics is needed [105, 106]. Below is a table of biomarkers in CRC;

Type of biomarker	**Use**	**Biomarker**
Risk	Assess cancer development	e.g *APC, SMAD4, MSH, MLH1, MSH6, PMS2, PTEN* and others
Screening	Cancer detection in asymptomatic population	Blood test, stool test (fecal hemoglobin)
Diagnosis	Detect presence of cancer	miRNAs, coloscopic mucosal staining
Classification	Phenotype stratification	MSI, CIN, CIMP
Prognosis	Outcome not related to therapy	MSI,miRNA,Coloprint,Oncotype DX and others
Prediction	Predict response to therapy	EGFR, *KRAS, BRAF, PTEN, TP53,* miRNA, MMR, HER3? HLA-A*02? Panel?

Table 5. CRC biomarkers in different areas.

Many biomarkers have been suggested but so far only *KRAS* and MSI are used as predictive biomarkers for the decision of therapy.

In recent years, complete cancer genomes, trascriptomes and exomes have been sequenced [106]. Epigenetic mechanisms like DNA methylation profiles have been used for CRC subgrouping and miRNA have been showed to play a role in CRC [106, 107, 108]. Development of the molecular techniques and target oriented research have increased understanding of the human genome and its complexity as well as cancer specific aberrations. Micro RNA´s are short RNA´s that bind to complementary mRNA molecules, hindering translation to protein.

The non-protein coding transcripts of DNA where miRNA (which modulates cellular processes) originate from have been conserved throughout evolution [107]. Micro RNA´s circulate in the blood and certain types of miRNA and changes in levels have been associated with tumor burden and cancer progression [108]. Comparing tumor exomes in individual patients can now identify cancer-driving mutations and sequencing differences between tumor and metastases providing information about genes important for the metastatic process [106].

Stage II, III and biomarkers

Meta analyses have clearly shown a better prognosis of MSI colorectal cancers compared to CIN [109]. The outcome of patients with CRC of stage II and III disease is difficult to predict. Over expression, amplification and mutated HER complex members that signals through the MAPK and PI3K pathways are common in colorectal cancer and therefore a good target of treatment. EGFR inhibiting drugs are therapeutic alternatives to chemotherapy in mCRC. However, when *KRAS* is mutated and constantly active as well as the MAPK pathway, downstream of EGFR, this will result in failure of EGFR inhibition. Further, only 30% of the wild-type *KRAS* patients will respond to EGFR inhibition. This indicates that there are other upstream or downstream effectors involved. A panel of predictive markers for stratifying treatment decision might be a way to proceed. Also individual tumor sequencing is interesting as well as an individual comparison of healthy genome to tumor genome [105]. In the future clinical setting, these parameters can be examined further as a step forward in personalized prognostics and prediction in CRC of stage II and III.

HER3

The human epidermal growth factor receptor type 3 (HER3 or human ErbB3) is a transmembranous tyrosine kinase receptor belonging to the HER complex. HER3 is embedded in the plasma membrane of the cell and neuregulins are its extracellular ligands. This receptor regulates cellular proliferation, differentiation, apoptosis and migration during embryogenesis and oncogenesis. The HER complex consists of four members, EGFR/HER1/ErbB1, HER2/ErbB2, HER3/ErbB3 and HER4/ErbB4 [110]. When a ligand binds, receptor dimerization takes place. While EGFR, HER3 and HER4 are activated by extracellular ligands, HER2 is an orphan receptor and is nonstop active [110]. The HER complex can be normally expressed, over expressed in an embryological manner and gene amplified or over expressed in cancer. A connection between the HER complex and the

WNT pathway might exist [111]. A challenge in imaging of HER3 is a quite low receptor expression in tumors, usually below 50,000 receptors per cell, together with significant HER3 expression in normal tissues [112]. Dysregulation of the HER complex is associated with a histological, malignant phenotype in CRC where the normal basolateral expression transforms to over expression in the complete cell membrane [113]. Receptors of the HER complex have an extracellular domain, an intracellular tyrosine kinase domain, which activate downstream signaling pathways, and an intracellular C-terminal tail (Figure 8).

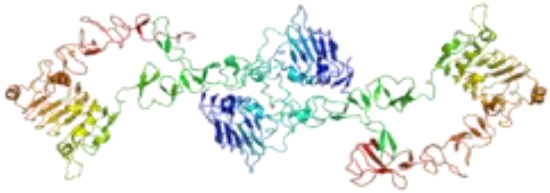


Figure 8. Protein structure of HER3.

Adapted from Wikiprofessional

However, HER3 has an inactive intracellular tyrosine kinase domain and its activation depends on the heterodimer formation with other HER complex members, preferably HER2 [114]. The HER2-HER3 unit activates downstream signaling pathways, such as P13K/AKT and RAS/MAPK and is considered as one of the most potent heterodimers in tumorigenesis(Figure 9) [115].

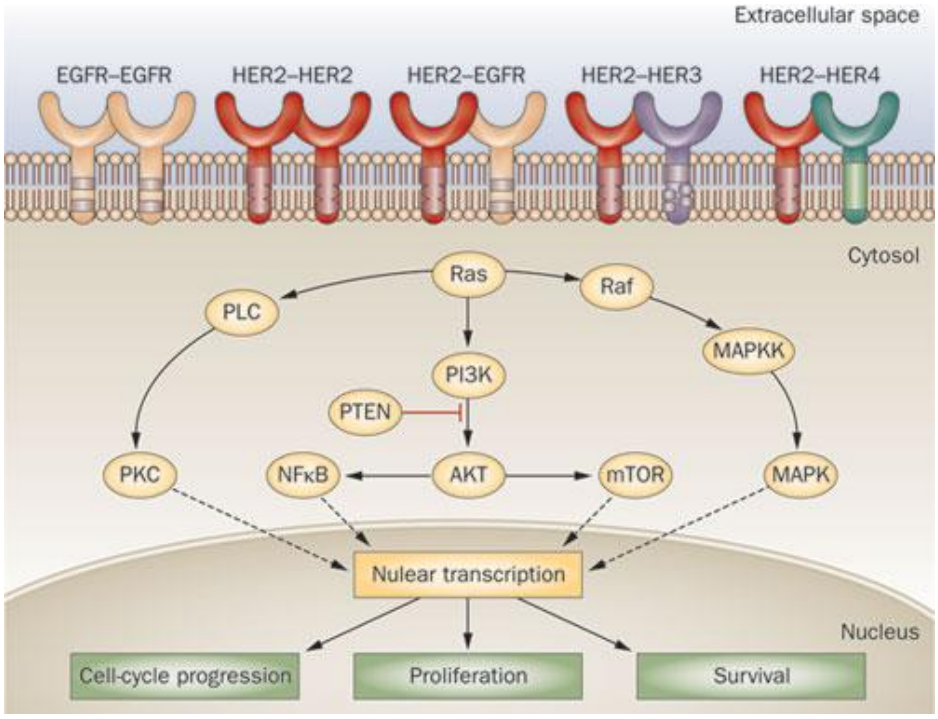


Figure 9. HER3 expression, dimerization and function.

Adapted from Nature Reviews Cancer

Evidence exists on the involvement of HER3 in various cancers, such as breast, prostate and colorectal cancer and its role in resistance to tyrosine kinase inhibiting (TKI) therapies [116]. The HER3 receptor is over expressed in about 70% of colon tumors [117, 118, 119]. Signaling can be affected by interdependency in between the HER complex (cross talk) [110]. For example, in response to trastuzumab (HER2 inhibitor) in HER2 over expressing breast cancer, HER3 can become up regulated and increase the signaling ability of HER2 as a compensation for its inhibition, which causes resistance to therapy [120]. It is also demonstrated that autocrine activation of HER2 with heregulin occurs through dimerization with HER3 in colon cancer [121].

The importance of HER3 in several cancers points to its potential as a molecular target in anti-cancer therapy. This has stimulated the development of appropriate pharmaceuticals [122, 123]. Currently, several HER3-targeting monoclonal antibodies are evaluated in clinical trials. To make anti-HER3 therapy effective, patients with HER3 over expressing tumors have to be identified.

Regarding the prognostic value of HER3 expression in colon cancer, the significance is not completely evaluated. Several reports have shown that HER3 expression is a prognostic factor in colorectal cancer (CRC) and might be of predictive value when cetuximab is given [124]. HER3 is found to be over expressed in primary colon cancer, rectal cancer, lymph node, liver metastases and it holds a certain prognostic value [117, 119, 125]. Studies indicate that the HER complex is a promising target for immunotherapeutic interventions with antibodies (TKI´s) and T-cells based approaches [124, 126].

MMR

Microsatellite instability (MSI) is an epigenetic or genetic instability found in sporadic colon and rectal cancer. Deficient MMR is a type of MSI where DNA errors are not repaired which result in a non-functional protein and in genomic instability. The deficiency or proficiency of MMR reflects the mechanism while MSI is the effect. Microsatellites are repeated units, one to six nucleotides long, usually located in non-coding regions and scattered throughout the genome and these sequences more frequently get errors. Deficient MMR tumors gain or lose repeat units at a higher frequency than in normal epithelial cells in the colon and rectum. Widespread MSI usually indicates mismatch repair deficiency (dMMR) [109]. The MMR genes maintain the stability and integrity of the genome. Inactivation of these genes promotes

accumulation at high speed of tumoral mutations. MMR genes are *MLH-1, MSH-2, MSH-6* and *PMS-2* (Figure 10) [127].

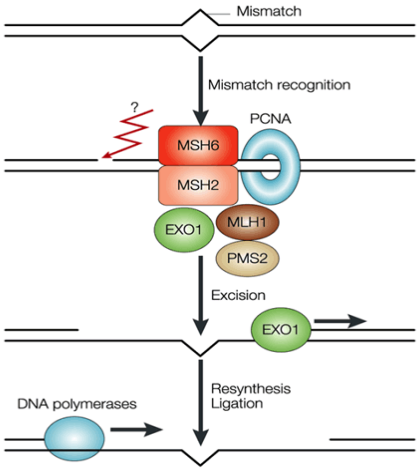


Figure 10. MMR, genetic function and involved genes. *Adapted from Nature Reviews Cancer*

Deficient MMR and MSI tumors characterizes a highly immunological subtype in CRC. This phenotype presents with topical immune growth control (raised number of cytotoxic tumor infiltrating lymphocytes), proximal colon location of tumor, female gender, high-grade tumor, apoptotic behaviour, low number of distant metastases and is of importance to inflammatory response and cancer control [127]. MSI is observed in several types of cancers; endometrial, ovarian, urothelial and prostate [128]. CRC and prostate cancer are linked by heredity [129]. MSI is used as a biomarker for prognosis and can predict sensitivity to 5-Fluorouracil (5-FU) in colon cancer [130, 131]. Moreover, MSI reveals genetic heterogeneity and expresses frame shift mutated proteins, a unique pool of tumor antigens that can act as T cells mediated immunotherapy targets and might have an immunological connection to HLA-A*02 (Figure 11) [132, 133].

HLA-A*02 genotype

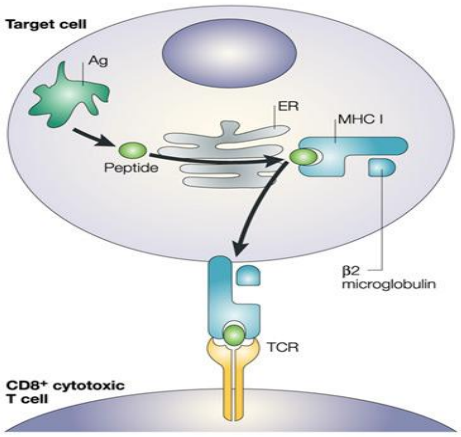


Figure 11. HLA and MHC presentation. *Adapted from Nature Reviews Immunology*

HLA´s are variant alleles, expressed in all cells of the organism. MHC is the product of a HLA gene. The HLA-A*02 genotype is one of the most frequent HLA-A alleles in all ethnic populations and the most common haplotype in Sweden (58%) [6]. Red blood cells (no nucleus) and neurons (low antigen presentation) have an explicably lower expression of HLA. Cells without HLA expression could be at risk for apoptosis via natural killer (NK) cells activated by absence of MHC class I. Cancer cells develop mechanisms to escape immune recognition. Certain HLA haplotypes are known to correlate to both risk of getting a cancer and prognosis in surviving cancer [133]. The loss or down-regulation of MHC class I has been studied [81]. The different HLA haplotypes have been associated with patients' prognosis in several cancers e.g. melanoma and ovarian cancer [134]. The locus of HLA is situated at chromosome 6 and encodes for peptide-presenting proteins. This is the first step in adaptive immunity and protection against foreign pathogens or abnormalities detected in the cell and in the organism. T-cells recognize tumor cells through antigen presentation on cell surface regulated by human leucocyte antigen (HLA). HLA class I molecules, HLA-A, -B, and -C, generally present intracellular peptides. The variation of HLA´s is restricted to the exons. This polymorphism defines a group of peptides that can potentially bind to a specific MHC and respond to infectious or cancer agents [132]. HLA alleles might correlate to other biomarkers or predict response to oncological treatments. HLA´s have been studied in different tumors and in colon cancer [133]. When giving immune based therapies to cancer patients, the HLA phenotype is correlated with diverse outcomes [132]. Though, immunotherapies should not solely rely on HLA class I restricted CD8+ T-cells [132, 135].

Two biological hypotheses can be mentioned to justify the selection of HLA-A*02. One hypothesis is that having HLA-A*02 genotype reflects an impairment of the immune response to CRC, resulting in a more aggressive disease or that the HLA-A*02 genotype is important in late disease where we see the results [81]. The other hypothesis is that inherited "linkage disequilibrium", a cluster of HLA alleles can involve nearby located genes for carcinogenesis and immune response. HLA-A*02 are over-represented and is a strong negative prognostic factor in ovarian cancer [81]. Prognosticity of HLA-A*02 has also been reported in prostate cancer [136]. Its relationship to colon cancer is not known but HLA-A*02 genotype has through personal communication of unpublished work been suggested to associate to negative prognosis in females (Villabona et al. "Analysis of immune-related prognostic markers in colon cancer in patients randomized to surgery or surgery and adjuvant cytostatic treatment", submitted 2015). Patients with HLA-A*02 genotype and CRC might have a significant worse outcome, however the genotype is not a risk factor [81]. It is probably the combined effect of

having HLA-A*02 genotype and loss or down-regulation of HLA class I MHC, that explains the poor prognosis.

AIMS OF THESIS

The overall aim of this thesis is to explore the HER3 expression in colon and rectal cancer.

The more specific aims are:

To evaluate the extent of HER3 expression in primary CRC.

To evaluate the extent of HER3 expression in colorectal cancer metastases.

To evaluate impact of HER3 on prognosis in colon and rectal cancer.

To investigate if there are associations between HER3 expression and tumor phenotype, tumor location or outcome of adjuvant chemotherapy in colon and rectal cancer.

To evaluate the prognostic and predictive effect of combining biomarkers HER3, MMR and HLA-A*02 in colon cancer.

PATIENTS AND METHODS

Patients

In paper I, III and IV the tumors derived from 236 respectively, 521 respectively, 493 Swedish patients with radically resected colon and rectal cancer of stage II and III from 29 hospitals in Sweden (Figure 12). Surgery was performed between 1991 and 1997. The follow up ended in November of 2004. Surgical tumor specimens (FFPE) originated from an adjuvant Nordic trial (n=2224) where patients (≤75 years) with CRC were randomized to either surgery or surgery plus 5-FU based adjuvant chemotherapy [33].

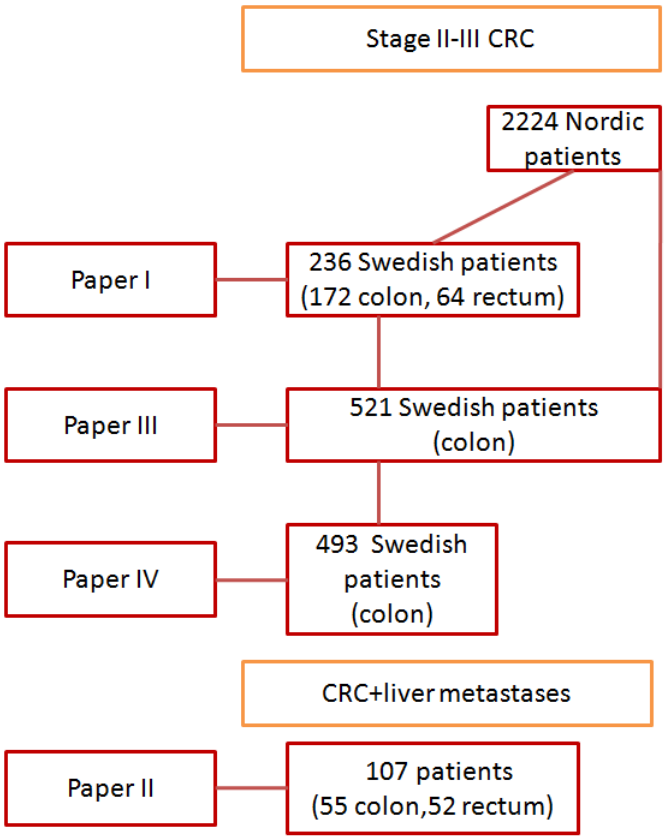


Figure 12. Flow chart of patients in paper I-IV.

In paper II, 107 patients with CRC and liver metastases were included (Figure 12). These patients all had resected primary tumors and liver metastases. The patients in this study were derived from a population-based cohort (n=255) undergoing liver resection for CRC metasta-

ses between 2004 and 2009 at the Unit of Hepatobiliary Surgery, Karolinska University Hospital. The primary CRC was resected between 1999 and 2009 in different Swedish Hospitals. The FFPE primary tumors (n= 107), corresponding nodal metastases (n=62) and liver metastases (n=107) were collected. Data from patient's medical records were retrieved.

The Dukes staging system and the stage 0-IV system for colon and rectal cancer were used in the pathology reports. The definition of proximal colon location was tumors in the caecum, ascending and transverse colon. The definition of distal colon location was the left flexure, descending and sigmoid colon. Regarding tumor differentiation, low-grade represents a well to moderately differentiated tumor while high-grade represents poor differentiated tumor. The definition of disease free survival was time from surgery to recurrence or to death of any cause. Clinical data in paper I, III and IV were retrieved from Regional Cancer Centers of Epidemiological Oncology in Sweden.

All studies of the thesis conform to the guidelines for prognostic tumor marker research, the REMARK criteria [104] and were approved by the local Ethical Committee at the Karolinska Institutet.

Immunohistochemistry (IHC)

IHC is demonstration of antigens within a tissue section by means of specific antibodies. The antigen-antibody complex is visualized by a mix of a secondary antibody, enzyme and a dextran chain which catalyzes a coloring producing reaction [137]. This technique is widely used for investigating tumor markers and was used in paper I-IV.

Immunohistochemical detection of HER3 and MMR expression was performed in primary colon or rectal tumors. HER3 expression was also detected in lymph node and liver metastases. The examined tumor specimens were derived from FFPE in 4-µm slices. A two-step procedure is described in text below and can be seen in Figure 13 and 14.

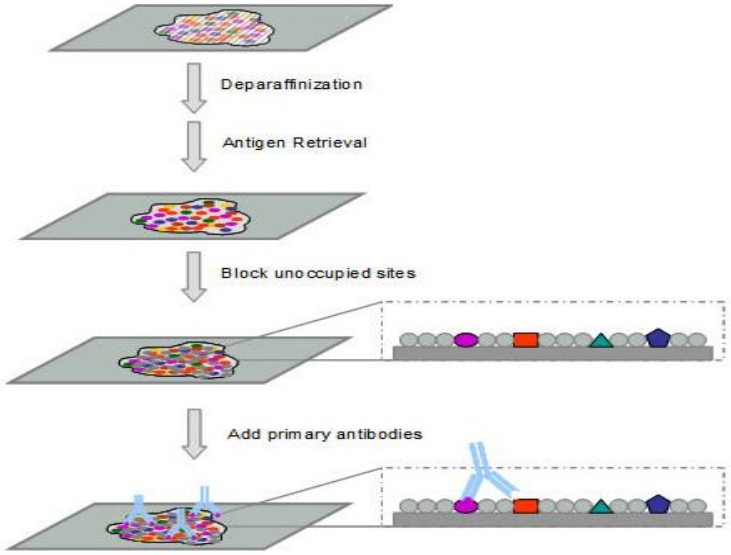


Figure 13. First step of IHC.

In step one, tumor sections were deparaffinized in xylene, rehydrated in ethanol and washed in distilled water. For antigen retrieval, slides were boiled in Dakos Retrievel (pH 9.0) in a pressure cooker (2100 Retriever) and rinsed twice. Endogenous peroxidation in the tumor was quenched by treatment with 3% hydroperoxidase. Background staining was reduced by incubation in 10% goat serum for 30 minutes. A HER3 monoclonal antibody from rabbit (Abcam, SP71 ErbB, ab 93739, dil 1:800) and to detect MMR proteins, MLH-1 respectively MSH-2, a mouse immunoglobulin G monoclonal antibody clone G168-15 (BD PharMingen, San Diego, CA, USA, dil 1:100) and clone FE11 (Oncogene Research Products, Boston, MA, USA, dil:1:100) were added and left over night at +4°C (Figure 13).

For step two, samples were rinsed and incubated with an amplification system, EnVision™, HRP system (DakoCytomation) for 30 minutes (Figure 14). Visualization of staining with 3,3´-diaminobenzidine tetrahydrochloride (DAB, DakoCytomation) was carried out followed by Mayers Haematoxylin staining.

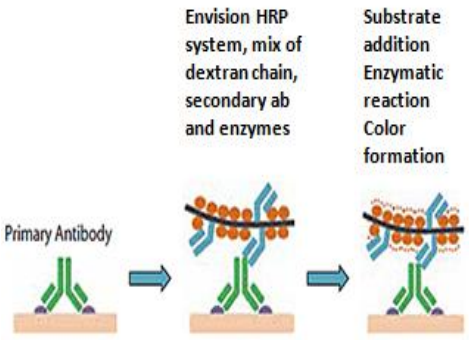


Figure 14. Second step of IHC.

Scoring

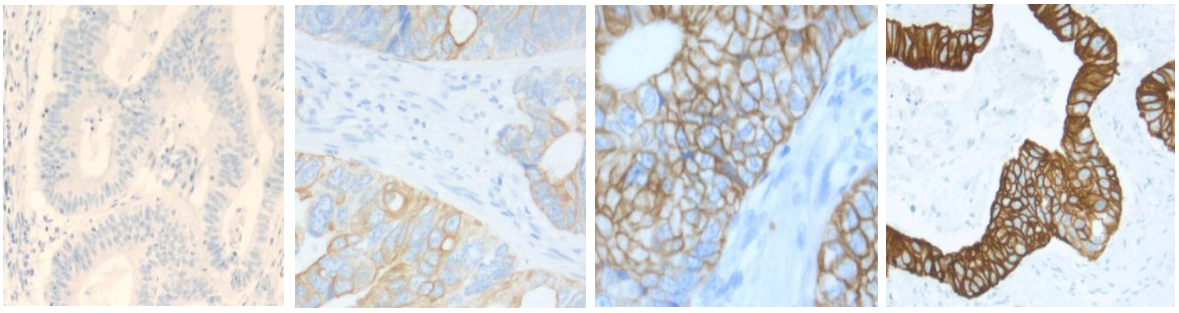
Slides were evaluated using a light microscope by two independent observers blinded to clinical and pathological data (F.L or K.Ö and M.H). Disagreements (<5%) were reviewed followed by conclusive judgement. The entire section of tumor was evaluated and an approximation was done on the percentage of stained cancer cells.

HER3

A representative tumor material of good quality and a well working antibody system for staining resulted in very few IHC failures. The HER3 receptor is mainly expressed in the cell membrane and only a small fraction is present in the cytoplasm [119]. Therefore, grading was done of the membrane.

The intensity of the membrane stain in cancer cells was graded 0-3. Absent stain or occurrence of staining of any grade in <10% was categorized as grade 0. Very faint membrane stain, present in parts of the membrane, was considered grade 1. Weak to moderate stain that was complete (circumferential) in the cell membrane or basolateral, was grade 2 and strong stain, complete or basolateral was defined as grade 3 (Figure 15). Cytoplasmic or background staining was not graded.

Grades 0-1 were categorized as low expression and grades 2-3, as high expression of membranous HER3. Staining pattern was scored according to the Hercept test, DAKO interpretation manual for gastric cancer since no established scoring of HER3 in colorectal cancer exists.

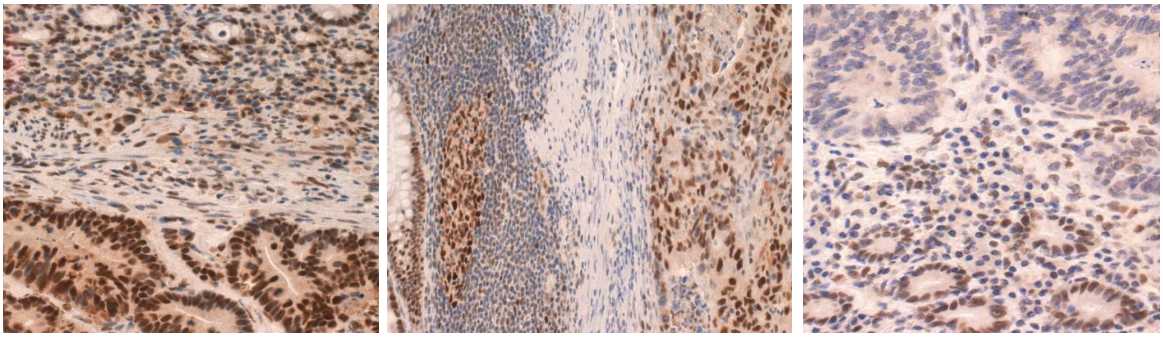


HER3, Negative (x10) HER3, Grade 1 (x20) HER3, Grade 2 (x40) HER3, Grade 3 (x20)

Figure 15. IHC in primary colorectal tumors of HER3 expression, Grade 0-3. Grade 0-1 are considered low expression and grade 2-3 as high HER3 expression.

MMR

A case was negative for expression of a given protein (dMMR) if the tumor cells displayed a complete absence of nuclear staining with the respective monoclonal antibody. Intact nuclear staining of normal tissue (lymphocytes and non-neoplastic stromal cells) close to the tumor was used as an internal positive control. The MSH-2 antibody showed a nuclear staining that was easy to interpret. The MLH-1 antibody yielded a few cases with patchy, heterogenous staining. If nuclear staining existed in the tumor, a case was considered as positive (pMMR). Figure 16 shows the IHC of MLH-1 and MSH-2.



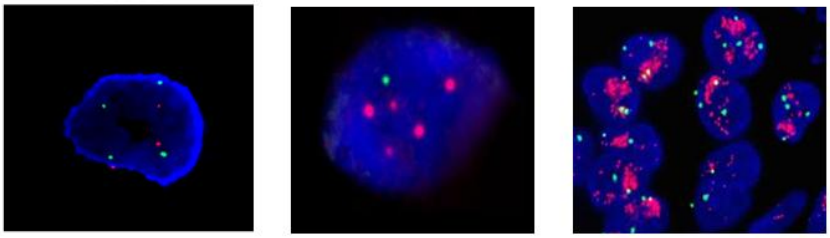
MLH-1 positive (pMMR) MSH-2 positive (pMMR) Negative (dMMR)

Figure 16. IHC in colon tumors of positive and negative stained tumor regarding MLH-1 and MSH-2. Magnification x 20.

FISH

Fluorescence in situ hybridization (FISH) was performed in paper I to clarify if HER3 gene amplification or ploidy occurred associated to high HER3 expression. FISH was performed in 58 tumors of all grades (a majority of grade 3).

HER3 detection was done on cut FFPE tumor sections according to standard techniques. After application of the HER3 probe (Zytolight HER3/CEN 12) the sections were treated with HyBrite programme and finally fluorescent stain (DAPI) was added. Microscope fluorescence scoring was done with Axio Imager Zeiss using filters for DAPI. Each color was recorded and processed digitally. Hybridization signal from the stained chromosomal region of HER3 lit in orange and the hybridization control signal from chromosome 12, lit in green. In normal cells, there are two HER3-regions (orange) and two chromosome 12 signals (green) (Figure 17).



Normal Ploidy Gene amplification

Figure 17. FISH

Polymerase chain reaction

In paper IV, HLA-A*02 genotype was assessed by doing PCR which is a method to amplify DNA. When few genes are analyzed in many samples, this is a preferable method.

DNA was extracted from the same FFPE tumors that were analyzed for HER3 and MMR expression by IHC. A High Pure DNA extraction kit (Roche, Molecular Biochemicals, Mannheim, Germany) was used, according to manufacturer´s protocol but omitting the DNase treatment.

The presence of HLA-A*02 was determined by genomic amplification with primers specific for HLA-A*02 (forward A*577LL and reverse A*503invLL). These primers are specific for all HLA*A02 alleles except A*020109, 0248, 0250, 0255 which are uncommon in the Swedish population. To exclude false negatives due to unamplifiable DNA, all negative samples were subjected to amplification of the housekeeping gene S14. Samples negative for S14 were excluded. The technique is described in detail in the article by Gamzatova et al [134].

Statistics

Statistical analyses of the results of paper I-IV were performed using STATISTICA software release 10 (StatSoft®, Tulsa, Oklahoma, USA). For HLA-A*02, HER3 and MMR analyses in paper IV, StatView for Windows (SAS Institute Inc. Version 5.0.1) was also used. In all studies, the χ^2 test was performed to examine relationship between patient´s demographics, tumor characteristics and HER3 expression. In paper I, the Spearman Rank´s correlation test was performed for the assessment of correlation of HER3 expression comparing two different sections of the same tumor. The same test was used in paper I and II to see correlation be-

tween HER3 in primary CRC and the corresponding metastases. Survival analyses were performed in all papers using the Kaplan Meier method and differences in survival were tested with the log-rank test. In all papers, Gehan-Wilcoxon test and Cox regression was used in the univariate analysis to examine correlation between HER3 expression, patient and tumor characteristics for overall survival (OS). Multivariate analysis was performed using Cox proportional hazard regression models in papers I, III and IV. The results were considered significant if $p<0.05$. All graphs in articles I-IV were made in STATISTICA.

RESULTS AND DISCUSSION

HER3 expression in primary tumor

The HER3 expression in primary CRC tumor was hypothized to be elevated.

In paper I and III, primary colon and rectal tumors of stage II and III were examined regarding HER3 expression. In paper II, CRC with liver meteastases were investigated related to HER3 expression. HER3 was detected at high frequency in the cell membrane in colon and rectal tumors. The distribution of HER3 expression of grades 0-3 and high and low HER3 expression can be seen in Table 6.

	Number of patients Study I, n=236	**Number of patients Study II, n=107**	**Number of patients Study III, n=521**	
HER3, Grade 0	67 (28%)	14 (13%)	153 (29%)	Low expression 30/20/33%
HER3, Grade 1	5 (2%)	7 (7%)	21 (4%)	
HER3, Grade 2	65 (28%)	47 (44%)	166 (32%)	High expression 70/80/67%
HER3, Garde 3	99 (42%)	39 (36%)	181 (35%)	

Table 6. HER3 expression assessed by IHC in primary colon and rectal tumors, study I-III.

In study I, which was a pilot study, 70% of primary tumors of colon and rectal cancer had a high HER3 expression (grade 2-3). When dividing tumor types, high expression was 63% in colon cancer and notably, 87.5% in rectal cancer. In 47 tumors HER3 expression was analyzed in two different areas. The HER3 expression was found to be homogenous and a strong correlation between paired samples was seen ($r=0.9$, $p<0.0001$).

A complementary FISH analysis was done in 58 tumors, with different grades of expression. We did not find any gene amplification of HER3 but in three tumors of grade 3, we found triploidy or tetraploidy. FISH was done to compare with HER2 that regulates through gene

amplification. HER2 does not have a known ligand like HER3 and HER2 are dependent on gene amplification to function in cancer.

HER3 seems to work through variation of expression. Over expression of HER3 is observed in several cancers but relevance of HER3 somatic mutations in oncogenesis is not completely established. Large scale genomic studies reported HER3 mutations in 11% of colon tumors [138]. Functional characterization of the HER3 mutants show that, together with HER2, they promote oncogenic signaling in a ligand independent manner. The role of HER3 as an obligate partner is well established in some tumors e.g. breast and is currently evaluated in other malignancies [116, 121].

Study III was an explorative, retrospective study which was enlarged and refined from the pilot study. Exclusively colon cancer of stage II and III, related to HER3 expression was included. High expression of HER3 (grade 2-3) was seen in 67% of colon tumors. In study II, an analysis of CRC with liver meteastases was done and the HER3 expression in the primary tumor was 80%. A higher grade of expression in CRC with liver metastases can be explained by that we examined a selected group of patients that all had liver metastases, a majority had lymph node metastases and a few had lung metastases. This cohort might represent a more aggressive and advanced disease.

When we initiated expression studies of HER3 in colon and rectal cancer, the literature was not conforming regarding expression [118, 119, 139]. In several reports, cytoplasmatic and membranous staining were graded and colon, rectal cancer and mCRC were sometimes mixed.

We have used immunohistochemistry to detect HER3 and it is a widely used technique in clinical praxis. However, it is a semi quantitative method to detect proteins and it is to some extent dependent on the investigators. Validation of our results using another quantitatively method (Western blot or mass spectrometry) is recommended but tend to require fresh or fresh frozen tumor. The protein quantification methods of HER complex have some drawbacks but have been done in CRC cells for HER3 [119, 140]. The discrepancies in expression of HER3 in CRC observed in different studies can be related to multiple causes, i.e. different staining protocols, antibodies, fixation time and variation in scoring methods (Rajkumar score, Hercept score, other). A weakness in our study is that tumors were collected from several hospitals and specimen routines might differ before paraffin embedding. The original histopathology reports were used but a pathologist review has been done.

The IHC scoring and validation of HER3 have not yet reached consensus. We have used the Hercept score of gastric cancer as guidelines for grading membrane intensity and change of pattern from a basolateral staining to a pathological total loss or circumferential staining. It has been easy to interpret the histology of HER3 and the used antibody has been working well. The 10% cut of has been chosen according to literature and is suitable for experimental expression analysis but in a clinical setting, it should probably be raised. IHC is a good technique, which is easy and samples from routine histology can be used. Compared to TMA, IHC, the whole slide has been analyzed which is a more accurate way if tumors are heterogenous. A comment on grading is that grade 1 constituted of very few cases and could probably be included in grade 0. The distinction between low and high HER3 expression was easy to interpret and is recommended for use in a clinical setting.

HER3 expression in colorectal metastases

HER3 expression in primary tumor and metastases was hypothized to correlate.

In paper I, HER3 expression in corresponding lymph node metastases was analyzed from patients with CRC of stage III. There was a high HER3 expression (grade 2–3) in 75% of the lymph node metastases and a correlation existed between HER3 expression in primary tumor and in corresponding lymph node metastases ($r=0.6$, $p<0.0001$). In paper II, liver metastases were synchronous in 57% of patients and a majority had metastatic lymph nodes at the time of primary surgery. All patients had liver metastases of which 82% presented high HER3 expression. The expression in lymph node metastases was 81%. HER3 expression in primary tumor correlated with expression in corresponding lymph node metastases ($r=0.65$, $p<0.001$) and in liver metastases ($r=0.45$, $p<0.001$). A correlation of HER3 expression between the two different metastases was seen as well ($r=0.65$, $p<0.001$). A comparison of the 107 patients in study II to the original cohort was done and survival was the same, indicating no severe skewness.

In study II, we have monitored HER3 through cancer progression. A selected group is examined that probably represents a more biologically aggressive disease. When separately analyzing the subgroups of patients with synchronous versus metachronous liver metastases regarding HER3 expression in the primary tumor related to survival, no difference was found although that could have been expected. This may be due to the relatively small sample size or to actual lack of biological difference.

It has been shown that most genetic aberrations in primary tumors are maintained in the CRC liver metastases [90]. Synchronicity of primary colon tumor and liver metastases might consitute a grave cancer disease but whether there are differences between primaries of synchronous and metachronous groups remain undetermined [88, 89]. When comparing our finding of maintained HER3 status in CRC metastases, it is in analogy with Miglio et al, concerning *KRAS* mutational status, which is maintained in corresponding CRC metastases [90]. One can suppose that there is an underlying oncological advantage of the phenomena regarding HER3 and *KRAS*.

There is a risk of under staging when less than 12 nodes (metastatic and benign) are analyzed. In paper I and III, >11 lymph nodes were analyzed in only 16% of the tumors. In paper II, 63% of the patients had >11 lymph nodes analyzed. This is a possible reason for under estimation of stage.

HER3 expression and prognosis in colon and rectal cancer

A high HER3 expression was hypothized to have a negative prognostic value in CRC.

In paper I, HER3 expression in the primary tumor was an independent prognostic factor for overall survival in the entire group of patients (p=0.026 resp. p=0.011, for OS and DFS). A high HER3 expression in the primary tumor was associated with worse clinical outcome and correlated with stage of disease. HER3 expression was a prognostic factor also in the subgroup of patients with stage II colon cancer according to OS (p=0.03) but not in colon cancer stage III. Neither did HER3 expression have a prognostic value in the small subgroup (n=64) of patients with rectal cancer. In the multivariate analysis, stage of disease, differentiation of tumor, age and HER3 expression in the primary tumor were independent prognostic factors for OS and DFS.

In paper II, there was no correlation between HER3 expression and OS neither using primary surgery, nor liver resection as a reference point. To comment on that, the number of patients in study II were low for analyzing prognosis and all patients developed liver metastases.

In paper III, we observed an independent correlation of distal tumors and HER3 expression related to DFS (p=0.03). In the multivariate analysis, a hazard ratio of 0.56 (low vs high HER3, 95% CI, 0.31-0.99) (p=0.047) was calculated. HER3 expression in primary colon tumor was not found to be prognostic for OS. Multivariate analysis showed that stage, age and number of analyzed lymph nodes ≥12 correlated independently to OS in the entire group of

patients. We also found in the univariate model that stage and sex correlated to DFS in distal colon tumors but only sex and HER3 expression remained significant after the multivariate analysis.

In study I, HER3 expression was prognostic regarding OS and DFS in the entire group of patients and in the subgroup of stage II colon cancer. These findings led to study III. Only colon cancer was included to avoid unexpected events of HER3 expression caused by radiotherapy. In study III, HER3 expression did not prove to be prognostic with respect to OS for the entire group of patients but prognostic for DFS in the group of distal colon cancers.

Why is HER3 expression in distal colon tumors prognostic regarding DFS but not for OS? DFS is an approved way of monitoring prognosis in colon cancer [32]. DFS has been used and validated in many adjuvant studies, which includes recurrence, all deaths and also secondary cancers. It is considered relevant to use DFS in studies even if the adjuvant treatment does not affect deaths other than of colon cancer. Plausible affecting factors are a low number of patients in the different subgroups and that the prognostic power of HER3 is not so strong.

High HER3 expression has in other studies been validated and found to be an independent prognostic factor with decreased OS in colon tumors. Furthermore, inhibition of HER3 in CRC cell lines induced apoptosis and reduced cell invasion and cell migration [119]. In a meta analysis of HER3 expression assessed by IHC, it was reported that it is a robust prognostic marker with correlation to worse survival in solid tumors [141]. Three CRC studies were included, two small studies showed that high HER3 was not prognostic for OS and one study showed that high HER3 was prognostic for shortened OS [119, 139].

HER3 phenotype, location and adjuvant therapy

HER3 expression in primary tumor was hypothized to associate with phenotype, colon location and maybe to adjuvant chemotherapy.

Location and grade

In study III, a high expression of HER3 was seen in 59% of proximal colon tumors versus 77% of distal tumors and in study I, the expression in rectal cancer was about 88% (Figure 18). The gradient of HER3 expression and association to tumor localization in the colon and

rectum is a novel finding in CRC. High expression of HER3 was associated with distal colon location ($p<0.0001$), independent of stage.

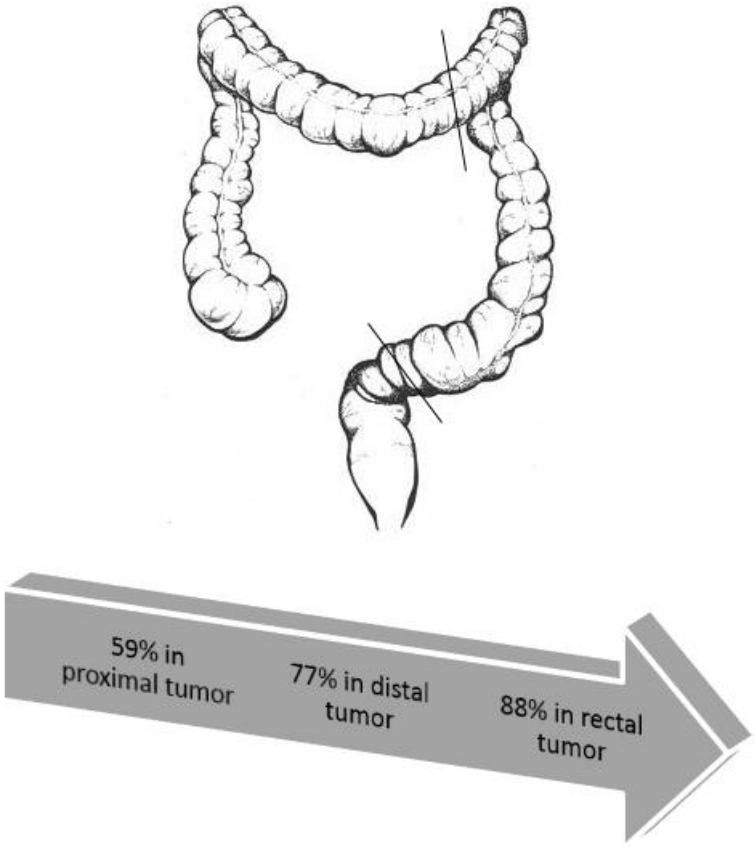


Figure 18. Distributional gradient of high HER3 expression related to proximal, distal colon and rectal tumor localization.

Regarding tumor grade, 71% of low-grade tumors expressed high levels of HER3 compared to 49% in the group of high-grade tumors. There was a correlation between low-grade tumor and high HER3 expression ($p<0.0001$) that was significant in the whole group of patients and in stage III but not for stage II. A higher amount of distal colon tumors was categorized as low-grade cancers 88% compared with 72% in proximal tumors, which was expected in a colon cancer materiel. Among low-grade tumors, there was a larger proportion of distal tumors with high HER3 expression (78%) compared with proximal tumors (64%). In multivariate analysis, we found that a higher frequency of distal colon tumors had a high HER3 expression compared with proximal, independent of tumor grade, OR=2.09 (95% CI, 1.39-3.14) ($p=0.001$). This means that we found that a higher number of distal colon tumors have high HER3 expression not only due to that distal tumors are more frequently low-grade tumors. This conforms to work by Missiaglia et al where expression of EGFR and HER2 correlated with distal tumor location [64].

Differences between proximal, distal colon and rectal tumors may have prognostic impact. Tumor location reflects biological heterogeneity that might have prognostic and predictive potential. The finding that high HER3 expression is more prevalent distally and decreases in high-grade tumors might associate inversely with e.g. MSI tumors presenting the relationship of proximal location and high-grade tumors.

In study II, expression of HER3 in the primary tumor did not correlate to any patient or tumor characteristics.

Adjuvant treatment

In study I, we found that patients with rectal cancer treated with pre-operative radiotherapy had a higher intra tumoral HER3 expression (97%) compared to non-radiated tumors (78%), independent of stage (p=0.023). HER3 expression might increase after radiation indicating that radiation itself raises expression. This is an interesting finding for using HER3 inhibitors after radiation in a neoadjuvant manner. However, this subgroup (n=64) is small and further research is needed to find out if pre-operative radiotherapy may have an impact on HER3 expression.

In study I-III, HER3 expression did not predict response to 5-FU based adjuvant chemotherapy. The study was not designed for this and the benefit of 5-FU based therapy in the original Nordic adjuvant trial was only 7% and did not reach significance [33].

Combining HER3, MMR and HLA-A*02

We hypothized that a combination of markers would improve prognostic and predictive value and associate to tumor location.

In paper IV, the HER3 and MMR expression were analyzed in primary colon tumors from 493 patients using IHC. 417 patients were analyzed for HLA-A*02 genotype with PCR.

HER3, MMR, HLA-A*02

Expression of high HER3 was seen in 66% and deficient MMR was found in 21% of colon tumors. The HLA-A*02 genotype was present in 58% of colon tumors. Deficient MMR was correlated to proximal colon (p<0.0001) and to high-grade tumors (p=0.01). To have a

dMMR tumor was prognostic for a better outcome in the entire group of patients regarding OS, which was expected. Female gender was associated to dMMR. There was a correlation between high HER3 and proficient MMR expression ($p<0.0001$). The HLA-A*02 genotype was not a prognostic factor in colon cancer. HLA-A*02 was like dMMR, associated to female gender. HER3 or MMR and HLA-A*02 did not correlate. Neither could correlation to localization of tumor be proven when combining HER3 or MMR and HLA-A*02.

Prognosis and prediction

Patients with high HER3/pMMR tumors had a worse outcome compared to the low HER3/dMMR group. The median OS for these patients were 81 months respectively 87 months. The adjusted hazard ratio for OS was 1.78 (CI 95%, 1.08-2.95) ($p=0.02$).

In patients with high HER3/pMMR tumors receiving adjuvant chemotherapy, a tendency was observed to extend DFS (median 84 vs 67 months) compared to the same group treated with only surgery, HR=0.75 (CI 95%, 0.54-1.04) ($p=0.08$). For OS, significance was not reached and no effect of adjuvant chemotherapy was seen in other groups.

In the small subgroup of high HER3/dMMR tumors, reduced DFS (median 30 vs 92 months) was observed when patients had a distal tumor (proximal vs distal; HR=0.10 (CI 95%, 0.02-0.47)) ($p=0.007$). The same was observed for OS.

In the subgroup of females with high HER3 expressing tumors of stage III who received surgery only, a decreased OS (median 40 vs 95 months) was seen if the patient had HLA-A*02 genotype, HR=0.37 (CI 95%, 0.14-0.94) ($p=0.03$). In analysis of the same subgroup of female patients with colon tumors of stage III, treated with surgery only, a shortened OS (median 38 vs 84 months) was observed if patients had the combination of pMMR and HLA-A*02 genotype compared to dMMR and missing HLA*02, HR=0.14 (CI 95%, 0.03-0.95) ($p=0.04$).

Univariate analysis showed that stage, tumor grade, number of analyzed lymph nodes and MMR ($p=0.02$) were significant factors for overall survival. In the multivariate analysis, stage, tumor grade, number of analyzed lymph nodes, MMR ($p=0.048$) and age remained significant.

Discussion

Combined biomarkers might offer an individualized approach in colon cancer that can separate a surgical curable cancer from a more aggressive disease and identify patients who will benefit from adjuvant chemotherapy. A single marker can be a part of the puzzle but lacks absolute power to constitute the whole truth of prognosis and prediction in cancer. The heterogeneity of a stepwise developing colon cancer spans through multiple molecular pathways. In study IV, we have analyzed markers from different sections of these pathways.

5-FU based chemotherapy is still golden standard in adjuvant treatment of colon cancer but administrative pattern has changed and additional given drugs differ. Even though the original adjuvant study and the tumor material are from the nineties, it is still useful for research.

MMR detection can be done with good accuracy using MLH-1 and MSH-2 with IHC [142].

In this study we have identified subgroups in which we can anticipate prognosis and observe a predictive tendency to adjuvant chemotherapy when combining HER3, MMR and HLA-A*02 genotype compared to each marker alone. A solitary biomarker or staging did not show a tendency of prediction regarding adjuvant chemotherapy in this tumor material before [33, 143]. High HER3/dMMR expression in a patient´s tumor correlated to distal location and resulted in reduced survival. However, this was observed in a small group of patients. When looking at patients with distal tumors and high HER3 expression alone in study III, a shortened DFS was detected. HER3 is highly expressed in both proximal (59%) and distal colon (77%) cancer and dMMR is mainly expressed in proximal colon tumors.

MMR is an accepted prognostic and predictive biomarker which is used in the clinic. We have analyzed our results independent from the effect of MMR status itself. The connection to female gender in colon cancer was known for dMMR and suspected for the HLA-A*02 genotype and might work through synergism when combined. The prognostic value of having the HLA-A*02 genotype in patients with colon cancer and association to female gender are novel findings (Villabona et al. "Analysis of immune-related prognostic markers in colon cancer in patients randomized to surgery or surgery and adjuvant cytostatic treatment", submitted 2015). A possible mechanism behind the prognostic trait of HLA-A*02, on a genetic level, may be found comparing male and female genetics.

Deficient MMR tumors are microsatellite instable which leads to generation of immunogenic neopeptides. The host antitumoral immune response is of high importance in cancer. Immune evasion caused by HLA class I impairment of antigen presentation can be seen in CRC. HLA

class II expression in CRC have been studied and in one third of patients with MSI tumors, HLA class II was missing [144]. Another mutated dMMR gene and protein, MSH-3, can cause an immunogenic frameshift sequence and different dMMR mutated proteins could act as targets for peptide based vaccines for therapeutic and preventive purposes [132]. Little is known on how HER3 expression in sporadic colon tumors interacts with MMR and HLA-A*02 genotype. HER2, is over expressed in breast cancer cells and act as targets for CTL´s. HER2 expression is reported to correlate to MHC class I and HER2 over expression might impair CTL mediated recognition of several HLA-A*02 restricted tumor antigens [83, 132].

Personalized medicine is to examine individual patients as well as subgroups of patients. It is important to pin point patients that do well and do not need unnecessary, extensive surgery or expensive, adjuvant chemotherapy which add suffering. It is equally important to identify patients that have a bad prognosis where the best available surgical and oncological treatments and extra surveillance are needed.

In paper IV, a combined analysis of HER3, MMR expression and HLA-A*02 status might improve prognosticity in colon cancer of stage II and III. Further studies are needed to find out if this combination can predict response to adjuvant 5-FU based chemotherapy in colon cancer or is of prognostic value for tumor location.

To conclude; In this thesis, we have shown that a majority of colon and rectal tumors and metastases over express HER3. There is increasing evidence that HER3 expression in colon and rectal cancer can be of importance as a predictive biomarker for biological therapy [124]. Anti HER3 agents are in the pipeline of new treatments and needs to be investigated further. Individual selection of therapy has been successful in HER2 expressing breast cancer by defining molecular subtypes that respond differently to treatments. This might be applicable for CRC, another solid tumor dependent on the HER axis [115]. When resistance to chemotherapy (5-FU+irinotecan) in mCRC occurs, a patient with HER3 expression in breast cancer can benefit from cetuximab treatment [116].

CRC is composed of different molecular subtypes and the detection of HER complex and HER3 can be of importance. Large, randomized studies will be needed to provide the evidence that will enable clinicians to determine optimal treatment of colon respectively rectal cancer patients.

CONCLUSIONS

In summary, the conclusions of this thesis are;

A high HER3 expression was found in 70% of primary tumors of CRC, stage II and III and in 75% of corresponding lymph node metastases. High HER3 expression was a negative prognostic factor for clinical outcome.

A high HER3 expression is seen in about 80% of CRC with liver metastases, in corresponding lymph node and in liver metastases. There was a correlation between HER3 expression in primary tumor and different metastases in CRC.

A high HER3 expression in colon cancer of stage II and III, was associated to distal colon location and low-grade tumor. High HER3 expression was of prognostic value according to DFS in distal colon cancer.

A combined analysis of HER3, MMR expression and HLA-A*02 can have a prognostic value and a tendency to prediction of adjuvant 5-FU based chemotherapy in subgroups of patients with colon cancer of stage II and III.

FUTURE PERSPECTIVES

Two aspects of HER3 expression in CRC and in corresponding metastases are interesting. One is that HER3, as a member of the HER complex and well-studied EGFR and HER2 expression, is not extensively studied in CRC. Further, knowledge of HER3 may definitely contribute to the complete picture of the HER complex and CRC. The second aspect is to look at HER3 as a prospect of drug development.

CRC is a disease of heterogeneous origins. As of today, prognostic and predictive biomarkers for CRC are few. A panel of markers selected because of biological hypothesis or biomarker screening with e.g. Tissue Micro Array (for gene-protein relationship) is suitable for CRC. Sequencing genes are quite easily done in this day and age, protein sequencing, is still a bit more complicated but can be done. Individual tumors and metastases can give us lots of information. In this way, the HER complex could be examnied in CRC.

The HER3 expression could be analyzed related to WNT signaling that has to do with shape, body axis and polarity of the cell as well as proliferation and migration. WNT receptors are evolutionary conserved and are present in all organisms. WNT signaling is important in embryogenesis and oncogenesis. Defect WNT is an early event in the adenoma carcinoma sequence [97]. The WNT receptor can be analyzed by IHC and also WNT gene products such as COX-2 and VEGF. HER3 expression and its connection to WNT might enlighten when and how normal HER3 distribution transforms to pathological distribution in a cancer cell. This eventual association could represent an early marker of CRC [145, 146].

HER3 expression related to the HER3 complex and EGFR inhibitors in mCRC is urgent to study since analysis of wildtype or mutated *KRAS* alone cannot manage the perfect patient selection. We have started a biobank of FFPE tumor slides from CRC patients and liver metastases who received modern, oncological treatment. Immunohistochemistry for prognostic markers have been done and can be continued in this material. Other techniques like PCR for HLA-A*02 can be done from these FFPE tumors and would be interesting to do in this CRC material since late stage cancer disease has been linked to HLA-A*02 [81]. In another collaboration, where patients with mCRC received cetuximab and irinotecan, we have analyzed HER3 expression with IHC but the data collecting is not yet complete. This direction of our HER3 research adds a modern and predictive dimension compared to this thesis where a gen-

eral mapping of HER3 expression in CRC has been done. Further, HER inhibitors are tested in clinical trials and it is going to be exciting to see in which tumor type HER3 inhibitors will be used first. Colon and especially rectal cancer are good candidates since HER3 is highly expressed in 70-88% of patients. A clinical trial with neo adjuvant, HER3 inhibitors, given after radiotherapy to rectal cancer patients would be intriguing to realize. It might create a new landscape of rectal cancer treatment, if the complete response rate can be adequately raised.

HER3 expression and immune response are sparingly studied in colorectal cancer as of today. Maybe HER3 like HER2 can be up and down regulated by HLA antigens and the HLA genotype may affect [126, 133]. Antitumoral helper T-cells can raise response in the HER complex in specific immunotherapies and HER3 expression in cetuximab treated patients is of interest [147]. Also the relationship between HER3 expression and CD8+ expression can be investigated further (detected by IHC and in a clinical setting, TMA-IHC).

Affibody Molecules

Affibody molecules are small, high affinity binding proteins based on a non-immunoglobulin scaffold. In a molecular imaging clinical study, a HER2 binding Affibody molecule have been shown to be a promising tool for the diagnosis of HER2 positive metastases in breast cancer patients [148] and currently, other Affibody molecules are entering clinical trials.

In vivo studies have shown specific uptake of high affinity HER3 binding Affibody molecules in HER3-expressing organs and *in vitro* studies have shown inhibition of HER3 signaling [149]. These findings may indicate a future potential of Affibody molecules as therapy of HER3 related cancers, including colorectal cancer.

The main challenge in HER3 imaging in cancer is the relatively low receptor expression in tumors and expression of HER3 also in normal tissue [150]. This may be partially circumvented by using a high affinity HER3 binding agent and/or pre-administration of a non-labeled agent. An advantage of Affibody molecules over antibodies for use in imaging include the rapid distribution and tissue penetration, as well as fast clearance of unbound molecule, providing high contrast images within hours (Figure 19).

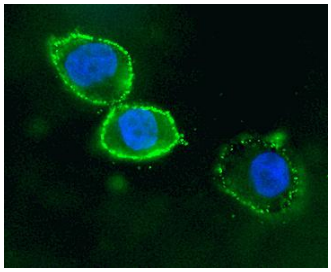
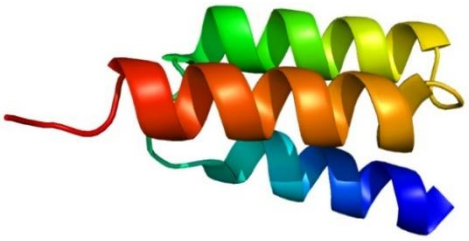
A  B 

Figure 19. A; Immunofluorescence staining of HER3 receptors expressed on the surface of adenocarcinoma cells with Affibody molecules (green fluorescence). ***Adapted from Affibody AB with permission.***
B; Ribbon diagram of the three-dimensional structure of an Affibody molecule.

For therapeutic applications, the small size and high solubility of Affibody molecules is beneficial, as it allows higher molar doses per volume, which also opens up for alternative means of administration. Moreover, Affibody molecules used as a therapeutic alternative to antibodies might bypass problems of acquired resistance observed for antibody based drugs.

Affibody molecules may be used to detect biomarkers and follow their actions in the tumor. One may also use them in mCRC to detect immediate and late effects of HER3 binding and inhibition of the HER complex. The high affinity of affibody molecules can be used in desirable, irreversible inhibition, which can reduce tumor cell growth [149]. Furthermore, immediate response of HER3 targeting and inhibition of a tumor in a clinical setting might be obtained with PET imaging compared to the delayed effects of chemotherapy and evaluating computed tomography.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Cancer är efter hjärt-kärl sjukdom den näst vanligaste orsaken till död i Sverige. Tjock- och ändtarmscancer kallas kolorektal cancer (CRC). Hos kvinnor är det den näst vanligaste tumörformen och hos män, den tredje vanligaste. Så är fallet både i Sverige och i ett globalt perspektiv. Vad vi äter, fysisk aktivitet, tobaksanvändning, alkoholintag, levnadsmiljö och ärftlighet är faktorer som kan inverka på om man får kolorektal cancer eller inte. Dessa faktorer är viktiga var och en för sig men kan också samverka och har betydelse även då sjukdomen brutit ut. Om man får en tumör i tjock- eller ändtarm är det viktigt att man opererar bort den. Prognosen är bättre om sjukdomen upptäcks tidigt i förloppet. Tilläggsbehandling i form av strålning, cellgifter eller antikroppar kan bli aktuellt beroende på sjukdomstyp. Även dottersvulster, metastaser i lunga och lever i begränsad form kan opereras bort idag. Cellgift får man för att döda enstaka cirkulerande tumörceller i kroppen. Med denna omfattande behandling förbättras prognosen och patienten kan till och med bli friskförklarad. Operation av en tumör och cellgift är gamla men fortfarande viktiga behandlingsprinciper. Det är ändå angeläget att hitta nya sätt att angripa och besegra cancer. Nya läkemedel med bättre och mer precis verkan och mindre biverkan behövs, särskilt då tumören spritt sig.

HER3 är ett naturligt protein som sitter på cellers yta. Här utövar den kontroll av olika funktioner som till exempel tillväxt. HER3 är en receptor vilket betyder att den kan ”öppnas” av en ligand och stängas, ungefär som ett nyckellås. Då det är öppet startas livsviktiga, kemiska reaktioner i cellen. Antalet receptorer på cellens yta kan öka och minska. HER3 ingår i en familj av 4 receptorer med liknande funktioner, vilka samarbetar inbördes. Tillväxt, kärlbildning och cellers rörlighet regleras av HER3 i fosterlivet, i normal tarm och i en tarmtumör. HER3 mönstret i cellen ändras mellan frisk och sjukt på ett karakteristiskt sätt.

I den här avhandlingen var syftet att studera förekomsten av HER3 i CRC samt i lymfkörtel- och levermetastaser. HER3 förekomst i CRC var då dessa studier inleddes, relativt okänd. Kartläggningen av HER3 används till att visa hur stor andel av CRC som har ett avvikande mönster och hur detta påverkar överlevnad och val av behandling. Vi har använt en teknik som benämns immunohistokemi för att kartlägga HER3. Man färgar HER3 med hjälp av en antikropp, en kemisk reaktion och färg. Tumörerna graderas i mikroskop i hög och låg förekomst.

Resultat och konklusion av denna avhandling blev att förekomsten av hög HER3 är 67% i tjocktarmscancer och 88% i ändtarmscancer. Om man har hög förekomst av HER3 i sin tumör, har man det sannolikt även i metastaser. Hög HER3 förekomst kan eventuellt kopplas till en sämre prognos men mer forskning och studier med fler patienter behövs innan man kan vara helt säker. HER3 förekomsten är högre i slutet av tjocktarmen och ändtarmen jämfört med i början, vilket inte var känt sen tidigare. I slutet av tarmen är därför förekomst av HER3 av större värde för prognos än i början. Vi har kombinerat HER3 förekomst med två andra potentiella markörer för CRC. Teorin bakom en kombination av markörer är att man ska kunna få en mer precis bedömning genom samverkan. Dessa heter MMR och HLA-A*02. MMR är viktig för cellers DNA reparation vilken ofta störs i cancer. HLA-A*02 är en gentyp som har med immunförsvaret att göra. Den skulle kunna inverka på så sätt att immunförsvaret inte bekämpar tumören som brukligt. Resultatet blir att en mer aggressiv tumör utvecklas än om man inte har gentypen. Med kombinerade markörer kunde patientgrupper med bäst respektive sämst prognos identifieras. De med sämst prognos ska då ha den bästa tänkbara kirurgin, onkologisk behandling och tät uppföljning. Så skulle sjukdomsåterfall och död kunna undvikas.

HER3 förekomsten är hög i CRC vilket gör den till ett utmärkt mål för skräddarsydd, individuell behandling. Eventuellt har även hög HER3 förekomst en negativ inverkan på prognos. Studier för att kunna blockera eller ”låsa” HER3 i tumörer och därmed förhindra cancerväxt pågår men än finns ingen medicin mot HER3 på apoteket.

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