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HISTOPATHOLOGICAL AND GENETIC ASPECTS OF COLORECTAL CANCER

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Han springer efter fakta likt en nybörjare på skridskor som dessutom övar sig på förbjudet område.

Franz Kafka

ABSTRACT

Colorectal cancer (CRC) is the third most common form of cancer in Sweden. The etiology of CRC is considered to be influenced by environmental risk factors on a background of constitutional and acquired genetic variations. It is estimated that inherited susceptibility accounts for approximately 35% of all CRC cases. The well-known high-risk syndromes familial adenomatous polyposis and Lynch syndrome, however, explain less than 5%. The remaining part of the "genetic" group is contributed by risk factors of much smaller magnitude, such as mutations in several low-risk alleles. Genome-wide association studies have identified multiple genetic loci and single nucleotide polymorphisms (SNPs) associated with an increased or decreased risk of CRC. Also, the histopathological profile of CRC shows considerable variation in relation to sex, age, tumor location, family history and mode of presentation, which could speak for different mechanisms of tumor development in different groups of patients.

The aim of **paper I** was to determine whether 11 newly identified genetic susceptibility loci were associated with tumor morphology, to confirm them as distinct and etiologically different risk factors in colorectal carcinogenesis. To that end, we analyzed 15 histological features in 1572 cases of consecutively operated CRCs during the years 2004-2006. Of the tested loci, five SNPs were significantly associated with morphological parameters such as poor differentiation, mucin production and decreased frequency of Crohn-like peritumoral reaction and desmoplastic response ($p=0.004$). The results are consistent with pathogenic variants in several loci acting in distinct CRC morphogenic pathways.

The aim of **paper II** was to provide a systematic histopathological characterization of CRC in the patient material above by comparing the morphology of tumors in men and women, in different age groups, in different anatomical locations, and in sporadic and familial cases, in order to isolate the effects of these four factors. Women had significantly more tumors with a high level of tumor infiltrating lymphocytes compared to men ($p=0.002$). Patients aged <60 years had less often multiple tumors but more often perineural invasion, infiltrative tumor margin ($p<0.0001$) and high AJCC-, T- and N-stage tumors ($p<0.0001$ for AJCC stage III) compared to patients >75 years. The results indicate that younger patients have a more aggressive disease. Most histological features showed a significant difference between left colon and rectum compared to right colon. Tumors in left colon and rectum were smaller and showed less often poor-, mucinous- or medullary differentiation or a circumscribed tumor margin ($p<0.0001$ for most features). Also, they were generally of a lower AJCC- and T-stage compared to right-sided lesions. The majority of features showed a gradient from right colon to rectum. The findings are in line with tumors in different locations having different genetic and embryological backgrounds as well as developing in different physiological settings. The only difference between the sporadic and familiar group was seen in vascular invasion which was more common among the familial cases ($p=0.012$).

The aim of **paper III** was to compare the clinicopathological profile of emergency and elective cases of CRC in relation to sex, age groups, location, and family history of CRC. In a multivariate analysis of 976 tumors from Stockholm County emergency cases more often showed multiple tumors, signet-ring cells, desmoplasia, vascular and perineural invasion, infiltrative tumor margin and high AJCC-, T- and N-stage tumors ($p<0.0001$ for several features). The findings could speak for emergency CRCs being an inherently different group of tumors with a more aggressive biology.

The aim of **paper IV** was to use the family history of cancer in 1720 patients with CRC together with genotyping and tumor morphology in order to find support for and define new CRC syndromes. There were significantly more cancers (other than CRCs) in the family history of the familial CRC cases compared to the sporadic CRC cases ($p<0.001$). There were also more bladder, prostate and gastric cancers as well as melanomas. One SNP, previously associated with both CRC and prostate cancer, was confirmed to be more common in families with CRC + prostate cancer. There were some support for different morphological profiles in four of the five tested syndromes with $p=0.010$ for an association between CRC + gastric cancer and Crohn-like peritumoral reaction.

LIST OF PUBLICATIONS

I. Ghazi S, von Holst S, Picelli S, Lindforss U, Tenesa A, Farrington SM, Campbell H, Dunlop MG, Papadogiannakis N, Lindblom A; The Low-Risk Colorectal Cancer Study Group.
Colorectal cancer susceptibility loci in a population-based study: Associations with morphological parameters.
Am J Pathol. 2010 Dec; 177(6):2688-93.

II. Ghazi S, Lindforss U, Lindberg G, Berg E, Lindblom A, Papadogiannakis N; The Low-Risk Colorectal Cancer Study Group.
Analysis of colorectal cancer morphology in relation to sex, age, location, and family history.
J Gastroenterol. 2012 Jan 18. [Epub ahead of print]

III. Ghazi S, Papadogiannakis N, Berg E, Lindblom A, Lindforss U; The Low-Risk Colorectal Cancer Study Group.
Clinicopathological analysis of colorectal cancer: A comparison between emergency and elective surgery cases.
Submitted for publication

IV. Forsberg A, Ghazi S, von Holst S, Björk E, Picelli S, Papadogiannakis N, Lindblom A.
Defining new colorectal cancer syndromes in a population based cohort of the disease.
Manuscript

LIST OF ABBREVIATIONS

APC	Adenomatous polyposis coli gene
BMP	Bone morphogenic protein genes
BRAF	v-raf murine sarcoma viral oncogene homolog B1
CIMP	CpG island methylator phenotype
CRC	Colorectal carcinoma
CRM	Circumferential resection margin
DCC	Deleted in colorectal cancer gene
ERβ	Estrogen receptor β
FAP	Familial adenomatous polyposis
FCCTX	Familial colorectal cancer type X
GWAS	Genome-wide association studies
IGF-1	Insuline like growth factor 1
KRAS	Kirsten rat sarcoma gene
LOH	Loss of heterozygosity
LS	Lynch syndrome
MLH1	Mut L homolog 1 gene
MMP	Matrix metalloproteinase
MMR	Missmatch repair
MRF	Mesorectal fascia
MSH2	Mut S homolog 2 gene
MSH6	Mut S homolog 6 gene
MSI-H/L	Microsatellite instability-high/low
OR	Odds ration
PMS2	Postmeiotic segregation 2 gene
RHPN2	Rho GTPase binding protein 2 gene
SMAD	Mothers against decapentaplegic homolog genes
SNP	Single nucleotide polymorphism
TGFβ	Transforming growth factor beta
TGFβR2	Transforming growth factor beta receptor type 2
TILs	Tumor infiltrating lymphocytes
TME	Total mesorectal excision
TP53	Tumor protein 53 gene
VEGF	Vascular endothelial growth factor

The names of genes are written in *italics* while their protein products are written in roman.

CONTENTS

1. INTRODUCTION TO COLORECTAL CANCER

Epidemiology

Colorectal carcinoma (CRC) represents almost 10% of all new cancers worldwide and ranks as the fourth most common cancer in men and third in women. The age standardized incidence varies at least 25-fold with high rates in industrialized, high-resource countries of Europe, Australia, New Zealand, North America and Japan (40-60/100 000) and much lower rates in other countries in Asia and Africa [1, 2]. Among immigrants and their descendants incidence rates rapidly increase up to those of their adopted countries, indicating that lifestyle, diet and environment are important risk factors [1]. Rates of rectal cancer are about 50% higher and rates of colon cancer about 20% higher in men than in women [3]. CRC is rare before the age of 40 years except in individuals with a predisposing condition. The incidence rate increases with age up to a peak in the seventh decade (mean age 60-65 years). The worldwide mortality rate is about half the incidence rate (608 000 deaths in 2002) and CRC is the fourth leading cause of death in cancer worldwide [4]. While the prevalence of CRC has increased over the last century, mortality rates have declined as a result of improved treatment, screening and surveillance [5].

In Sweden CRC is the third most common form of cancer in both men and women. It contributes to about 7% of all cancer diagnoses with approximately 5000 new cases per year and the lifetime risk of developing CRC in Sweden is 5-7% [6]. The relative 5-year survival for colon cancer diagnosed 1993-1995 in Sweden was 57% for men and 59% for women. The corresponding figures for rectal cancer were 54% and 60% respectively [6].

The prognosis of CRC is strongly correlated to tumor stage which is based on the depth of tumor infiltration through the bowel wall and the presence of lymph node or distant metastases. The 5-year survival is >90% in stage I, 75-85 in stage II, 45-60% in stage III and 0-5% in stage IV [7].

Etiology

The etiology of CRC is today considered to be influenced by environmental risk factors on a background of constitutional and acquired genetic variations. Based on studies of twins it is estimated that 35% of CRCs have a potentially identifiable genetic cause [8]. Among these are the well-known syndromes familial adenomatous polyposis (FAP) and Lynch syndrome (LS). These two conditions however explain less than 5% of all CRCs. The remaining part of the "genetic" group is contributed by risk factors of much smaller magnitude, such as mutations in several low-risk alleles, as has been shown in studies of CRC as a complex disease [9]. The genetics of CRC and the importance of family history for this disease will be dealt with in Chapter 2 and 3. Most CRCs are sporadic and occur in individuals over 50 years of age. These tumors develop as the consequence of

environmental carcinogenic exposure and secondary genetic or epigenetic events in somatic cells [10].

Traditionally, several risk factors associated with an affluent western lifestyle have been implicated in the etiology of CRC. These include a diet rich in calories and animal fat, a high consumption of red meat and processed foods as well as a lack of fresh fruit, vegetables and dietary fibre. Obesity, alcohol and smoking are also risk factors for CRC, while physical activity, dietary calcium supplementation, vitamin D, non-steroidal anti-inflammatory drugs and estrogen replacement therapy in women exerts a protective effect. The inflammatory bowel diseases (IBD) ulcerative colitis and Crohn's disease confer an increased risk of CRC, although there are varying reports regarding the cumulative risk.

Red meat and processed foods

Observational and prospective studies have shown an association between consumption of red meat and an increased risk of CRC [11, 12], although there is some inconsistency in the reports. Red meat, as well as processed meat, increases fecal levels of N-nitroso compounds, which are potentially carcinogenic. Some N-nitroso compounds have alkylating agent properties and have been demonstrated to induce changes in the *KRAS* gene which is activated in the oncogenic pathway to CRC [13]. Red meat also increases the level of DNA adducts in the epithelial cells of colon. These adducts are highly reactive agents that have been recognized as playing a central role in carcinogenesis [14].

Fruits, vegetables and fibre

Diets low in fruits and vegetables have been associated with an increased risk of CRC in observational studies [15, 16]. A high intake of fibre has been correlated to a reduced risk of CRC in some studies [17, 18], but not in others [19, 20]. In a systematic review of five studies it was concluded that there was insufficient evidence to state that increased dietary fibre reduced the incidence or recurrence of adenomatous polyps which are precursor lesions to CRC [21]. Proposed mechanisms for dietary fibre to reduce the development of CRC are decreased exposure of the colonic mucosa to carcinogens (by shortening the intestinal transit time) and the fermentation of fibre by colonic bacteria to produce short-chain fatty acids such as butyrate, which has been demonstrated to induce cell cycle arrest, differentiation and/or apoptosis in vitro [22].

Obesity

An elevated body mass index has been linked to the development of both colonic adenomas and CRC [23, 24]. Obesity is associated with the metabolic syndrome, behind which either the presence of insulin resistance or visceral adiposity is the driving force. In vitro studies have shown that insulin promotes cellular proliferation, inhibits apoptosis in colon cancer cell lines and promotes the growth of colorectal cancer in animal models [25]. Hyperinsulinaemia is associated with elevated levels of insulin-like growth factor 1 (IGF-1) which has been demonstrated to promote cell migration, cell proliferation and angiogenesis and inhibit apoptosis and cellular adhesion. Obesity also leads to a change in serum levels of adipocytokines such as leptin and adiponectin which in vitro have effect

on cell proliferation, angiogenesis and promotion of tumorigenesis and could therefore contribute to the development of CRC [26]. Visceral adiposity has been linked to a state of chronic low-grade inflammation and persistent activation of the nuclear transcription factor NK-κB with subsequent transcription of genes promoting tumorigenesis [27].

Physical activity

A number of potential mechanisms for physical activity to reduce the risk of CRC have been suggested, including decreased gastrointestinal transit time, altered immune function and the role of insulin and IGF-1 according to above [28]. High levels of insulin and IGF-1 are associated with low exercise levels. Interestingly, mutations in both *KRAS* and *TP53*, genes involved in the CRC pathway, have been linked to reduced levels of physical activity [29, 30].

Smoking and alcohol

There is currently insufficient evidence to establish a causal relationship between smoking and CRC, but prospective studies have shown an increased risk ratio among smokers for both colon and rectal cancer [31, 32]. It has been reported that smoking may be associated with particular subtypes of tumors, such as cancers showing p53 overexpression or transversion mutations in the *KRAS* gene [33].

Pooled data from cohort studies have showed an increased risk ratio of developing CRC in those drinking >45g alcohol/day [34]. It has been proposed that a decreased intake of folate, which participates in DNA synthesis, among patients with significant alcohol dependency could explain the higher risk of CRC in this group [35].

Ulcerative colitis

Ulcerative colitis (UC) is an inflammatory bowel disease of unknown etiology affecting children and adults with a peak incidence in the early third decade. CRC is a serious complication and accounts for 10-15% of all deaths in IBD patients. In different studies the cumulative risk for CRC after 20 years of UC varies from 1 to 34%. This wide range is probably explained by variation in age at diagnosis, gender, extent and duration of the disease as well as use of different patient populations. In a meta-analysis the risk of CRC was 2% after 10 years, 8% after 20 years and 18% after 30 years of disease [36]. The risk is highest for colitis involving the whole colon, while ulcerative proctitis is not associated with an increased risk. UC-associated cancers are often multiple and evolve from flat lesions through low-grade and high-grade dysplasia, or from raised dysplastic lesions (dysplasia-associated lesion or mass, DALM). The molecular alterations in UC-associated CRCs are similar to sporadic CRCs, but seem to differ in frequency and sequence. In contrast to sporadic carcinomas, *APC* and *KRAS* mutations occur late in the carcinogenic process, while changes in *TP53* occur early. 15% of UC-related carcinomas show a high level of microsatellite instability. In addition, oxidative stress, cyclooxygenas-2 (COX2), cytokines such as TNFα and IL-10, growth factors and gastrointestinal microbiota are thought to play a key role in the carcinogenesis of CRC in patients with UC [3, 37].

Gene-diet interactions

In brief, the molecular pathways that underlie the epidemiological associations are poorly understood because of complex interactions that may involve dietary patterns, nutrient composition of foodstuffs, food preparation techniques, hormonal effects, genetic characteristics and gene-diet interactions. In a meta-analysis to detect potential interactions between ten single nucleotide polymorphisms (SNPs) associated to CRC and selected risk factors including sex, body mass index, smoking, alcohol, dietary intake of red meat, vegetables, fruit and fibre, the only gene-environment interaction that was statistically significant was between one SNP and vegetable consumption [38].

Symptoms and signs

In its early stages CRC is usually asymptomatic. There is no good correlation between the duration of symptoms and tumor stage. The main symptoms are change in bowel habits, especially obstipation (sometimes alternating with diarrhea), and haematochezia. Associated abdominal distension and pain may follow. Right-sided tumors may produce less obstructive symptoms but present themselves with anemia, weight loss and impaired general condition. Left-sided tumors however tend to cause obstructive symptoms, change in bowel habits, haematochezia or mucus in stools. Rectosigmoid lesions can produce tenesmus and rectal bleeding. Impaired general status, vomiting, cachexia, ascites and anemia are signs of advanced disease [39]. 15-30% of CRCs present themselves as surgical emergencies, most often as obstruction with colon ileus or perforation [40, 41].

Diagnostics

The primary work-up of patients with suspected CRC includes medical history, family history, physical examination and colonoscopy. If the colonoscopy reveals a tumor, a computerized tomography of the abdomen and thorax should be performed in order to visualize any spread of the tumor. All patients with suspected or confirmed CRC should be referred to a surgical clinic where further investigation can be performed if necessary [39].

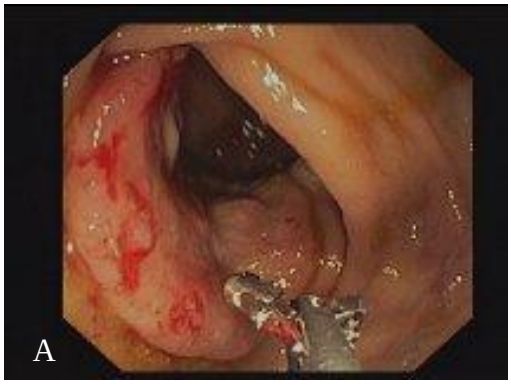
Colonoscopy

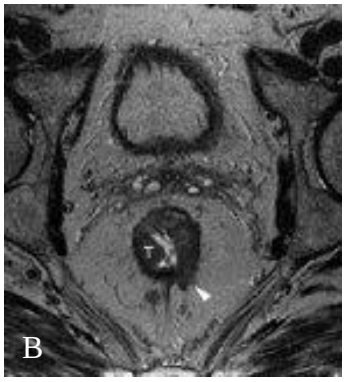
Regardless of whether a rectal tumor is found or not, a colonoscopy ought to be performed to exclude any synchronous tumor. Colonoscopy has an advantage over barium-enema and computed tomographic colonoscopy ("virtual colonoscopy") since it allows for biopsies to be taken (Figure 1A). In addition, the therapeutic removal of small lesions such as polyps by snare polypectomy or endoscopic mucosal resection is possible [39].

Transrectal ultrasonography

This method has traditionally been used to stage rectal cancer preoperatively since it allows an estimation of the depth of tumor invasion in the wall, especially among superficial tumors [42]. Regional lymph nodes may also be visualized, although transrectal

Figure 1. A. Picture from a colonoscopy showing an elevated plaque-like cancer. Biopsy forceps visible in the lower part. **B**. MRI of a rectal cancer**.** T and arrow indicates tumor.





ultrasonography cannot reliably separate metastatic lymph nodes from benign ones [43]. Due to this and the technical evolution of magnetic resonance imaging (see below) the latter method has largely replaced transrectal ultrasonography in the preoperative staging of rectal cancer.

Magnetic resonance imaging (MRI)

High-resolution MRI has been shown to be superior to both computerized tomography and transrectal ultrasonography for local staging of rectal cancer [44] (Figure 1B). It has the ability to differentiate tumor from the lamina muscularis propria and can delineate the mesorectal fascia (MRF) which forms the circumferential resection margin (CRM) at operation [45]. The presence of regional lymph node metastases can be assessed although the method still has its limitations [39].

Abdominal ultrasound (US)

This is the most common imaging method used to evaluate the liver for metastases. Preoperative examination shows synchronous liver metastases in 10-15% of CRC cases. Enhancement with contrast improves both sensitivity and specificity [39].

Computerized tomography (CT) and other methods

CT is an alternative to US in the search for liver metastases. With contrast enhancement this imaging modality has a higher diagnostic accuracy than US without intravenous contrast. CT is also an efficient method to detect metastases and recurrence after surgery [39] and is used preoperatively to screen for pulmonary metastases. Pulmonary X-ray is sometimes done preoperatively. Positron emission tomography (PET) and skeletal scintigraphy are used in selected cases to detect widespread disease.

Surgical treatment

Curative resection is the single most important factor for patient survival. Surgery is the primary treatment for CRC and can be done as either an open or laparoscopic procedure.

The latter is less common in Sweden where about 5% of rectal cancer operations are done with laparoscopy. Careful preoperative assessment of the extent of tumor spread, involvement of the MRF and TNM-staging is important. This is preferably done at multidisciplinary team (MDT) conferences where surgeons, radiologists, oncologists and pathologists discuss the need of preoperative radio- or chemotherapy, possible inclusion in any study and the type of surgery. Even if curative surgery is impossible due to metastatic disease it might be worthwhile to try to remove the primary tumor to relieve the patient from obstructive symptoms or bleeding. An alternative is to offer the patient chemotherapy and to evaluate the result after two to three months. If the response is good curative surgery might then be considered [39].

The aim of CRC surgery is to remove the tumor-bearing segment of the bowel with sufficient surgical margins as well as the mesentery and regional lymph nodes of that segment. Adequate removal of lymph nodes is important not only for postoperative TNM-staging but may also have therapeutic importance. Growth by the tumor onto adjacent organs can be difficult to distinguish macroscopically from fibrous or inflammatory adhesions. Even if there is local tumor involvement of the uterus, ovaries or loops of small bowel there might not be distant metastases why an en-bloc resection might still be curative. As in all curative oncologic surgery the aim is a free longitudinal margin of at least 10 cm. In rectal cancers operated with total mesorectal excision a much narrower distal margin is accepted because of the anatomical situation and the distance to the external sphincter (see below). For a well-differentiated tumor in rectum a longitudinal margin of 1 cm is considered sufficient, but a wider margin is desirable for poorly differentiated tumors. If a tumor is found to be fixed and not resectable at exploration one should refrain from attempts to remove it. Instead, after creating a loop stoma as a diversion, the patient should be referred to an MDT conference where a decision of neo-adjuvant treatment might be made [39]. Regardless of the type of tumor preoperatively suspected, the surgical procedure should be performed in a standardized way according to below.

Colon cancer operations

Right-sided hemicolectomy is performed for tumors located in the cecum, ascending colon, hepatic flexure or the right part of the transverse colon. The ileocolic and right colic vessels are divided and the right side of colon including the hepatic flexure and 10 cm of the distal ileum is resected (Figure 2). Recently, a more radical resection of the colonic mesentery and the lymphatic drainage in right-sided hemicolectomy has been presented and is becoming increasingly common. In this procedure, where the mesentery is removed intact (in analogy to total mesorectal excision,) a five year cancer related survival of 91% for stage II and 70% for stage III cancers has been reported [46].

Tumors in the transverse colon are usual operated as an extended right-sided or left-sided hemicolectomy if the intention is curative.

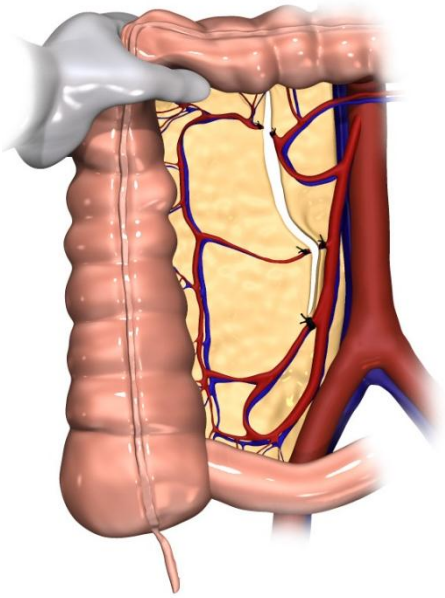


Figure 2. Schematic view of a right-sided hemicolectomy. The ileocolic and right colic vessels are divided with the mesentery. Illustration by Hanna Bringman.

Left-sided hemicolectomy is done for tumors in the left part of the transverse colon, hepatic flexure and the descending colon. In this procedure the inferior mesenteric vessels are divided proximally and the left colon including the splenic flexure is removed.

Sigmoidal resection is used for tumors in the sigmoid. However, nowadays left-sided hemicolectomy is preferred in most cases. For tumors close to the rectosigmoid junction a high anterior resection should be undertaken with a cylindrical resection of the mesocolon/mesorectum at least 5 centimeters below the distal margin of the tumor.

Subtotal or total colectomy might be considered when there are synchronous tumors in both left and right colon, if the patient suffers from FAP or LS or has any other type of strong risk factor for multiple CRCs. Ileorectal anastomosis is usually performed in these cases.

Emergency colon resections are common. 15-30% of CRC patients present themselves as emergency cases, most often due to obstruction (78%), perforation (10%) or bleeding (4%) [40, 41]. If the tumor is located in the right colon the same type of operation as in elective cases can usually be performed and a primary anastomosis can be created. The choice of operation for left-sided lesions however remains controversial. In these cases the bowel proximal to the obstruction is usually circulatory compromised and shows diastatic widening or even perforation according to the law of La Place. Depending on the status of the bowel proximal to the obstruction, several different surgical approaches, from subtotal colectomy to segmental resection, may be considered. A primary anastomosis might be combined with a temporary relieving loop-ileostomy to limit the effects of a possible leakage. In case of perforation, fecal peritonitis, steroid treatment or other high-risk factors for operation, the tumor should be resected, a colostomy created and the rectal stump usually left blind (i.e. Hartmann's procedure). If, however, the cecum is severely dilated, discolored or perforated a subtotal or total colectomy is advisable, even though it will affect the bowel function with frequent stools and possibly impaired fecal continence. In severely debilitated patients it might be wise to refrain from a primary anastomosis in favor of creating a double-barrel stoma. A method currently under

evaluation is stenting (i.e placing a short hollow plastic or metallic tube in the obstructed part of the tumor) during colonoscopy to keep the lumen open. This can be done either as a "bridge to surgery" or as a palliative procedure for inoperable patients.

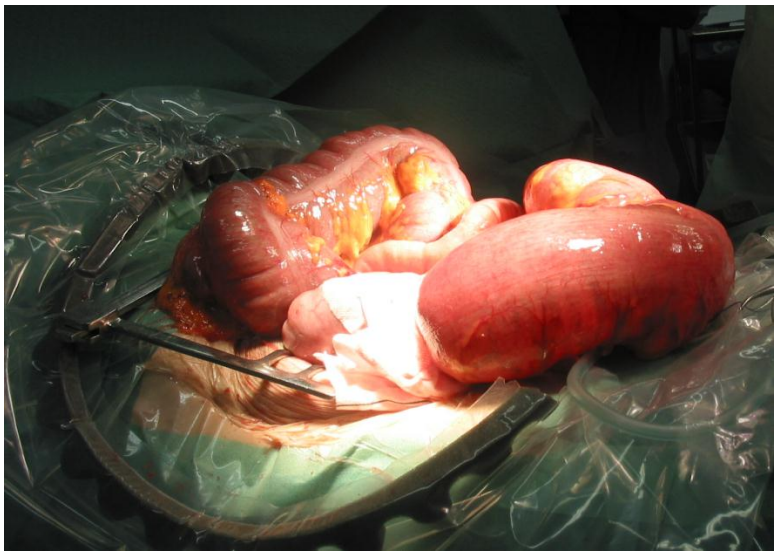


Figure 3. Emergency surgery for a left-sided colon cancer which has caused obstruction and subsequent dilatation of loops of small and large bowel.

Rectal cancer operations

Curative surgery for rectal cancer can be performed in basicly three ways: 1. Anterior resection with anastomosis, 2. Anterior resection without anastomosis (Hartmann's procedure) or 3. Abdominoperineal amputation of rectum. In addition, there are local, procedures such as transanal endoscopic microsurgery (TEM) that may be used for radical excision of smaller lesions.

Anterior resection with anastomosis is performed in 50% of patients and is the most common surgical procedure for rectal cancer in Sweden [47]. It is performed for tumors in the middle and upper rectum when a distal margin of at least 1 cm can be achieved [48]. If this is not possible an amputation of the rectum should be undertaken instead. In an anterior resection the rectosigmoid colon is mobilized, the pelvic floor opened and the inferior mesenteric artery ligated and divided. The tumor is removed according to the principle of total mesorectal excision (TME) which was introduced in 1982 by Heald. This technique involves a sharp dissection of the avascular plane between the mesorectum and pelvic structures down to the pelvic floor. The dissection outside the mesorectal fascia ensures a complete resection of the mesorectum belonging to the tumor-bearing part of the rectum (Figure 4) [49]. The introduction of TME has dramatically improved local tumor radicality with local recurrence rates usually between 3 and 11% today [50, 51]. After the excision, the remaining part of rectum is connected by a side-to-end anastomosis to distal colon or to a colonic reservoir. This can be done either hand-sewed or, more commonly, by using a circular stapling device. The frequency of clinically observed leakage from a low rectal anastomosis is 5 to 10%. Performing a temporary diverting loop-ileostomy has been recommended in patients with a low anterior resection to prevent pelvic sepsis [52].

Hartmann's procedure, which is performed in 10% of rectal cancer patients, is an anterior resection without anastomosis. An end-colostomy is created and the rectal stump is left blind. This operation is often performed on debilitated patients and patients with incontinence or poor preoperative anal sphincter function.

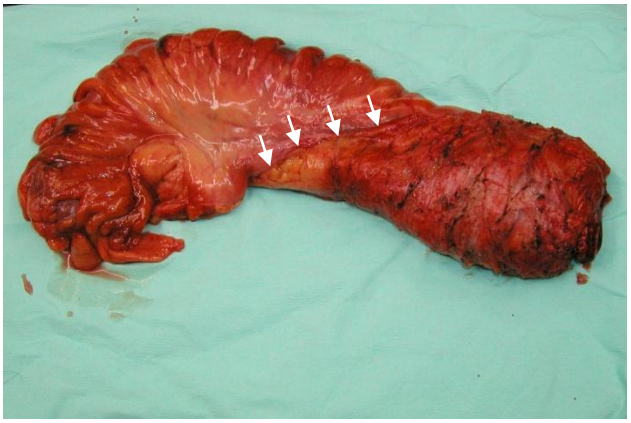


Figure 4. Specimen from a total mesorectal excision (TME) viewed from the right. The distal resection margin is to the right in the picture. Arrows indicate the border between the peritoneal reflexion and the mesorectal fascia.

Abdominoperineal amputation of rectum is a removal of the entire rectum, anal canal and anus. It is used in 80% of all patients with a low rectal cancer (i.e. 0.5 cm from the anal verge) to ensure an adequate distal resection margin. A permanent terminal sigmoid colostomy is created and the resection of the tumor follows the principles of TME all the way down to the pelvic floor. Abdominoperineal amputation carries a local recurrence rate of 23% [53], possibly because of the technical difficulties resulting in perforation of the tumor and positive resection margins. Recently the introduction of extralevator abdominoperineal resection instead of standard abdominoperineal resection might improve the outcome [54].

Screening for colorectal cancer

CRC fulfills most of the criteria for screening to be applied. The natural history is well known compared to many other cancers. CRC may be cured if detected early and even prevented by removal of possible precursor lesions such as adenomas. The development of CRC is usually slow (5-10 years), making screening for the disease attractive. Possible methods for this include sigmoideoscopy, colonoscopy, imaging and molecular stool testing. However, the only screening modality that has been subjected to adequate scientific assessment is fecal occult blood testing (FOBT). Randomized clinical trials have shown a mortality reduction of 15-18% after 10 years follow-up in those targeted for screening with Hemoccult test [55]. In a report from 2005 it was concluded that there is sufficient evidence for the effect on mortality of screening for CRC biannually with FOBT. There is, however, lack of evidence on the effectiveness of screening as a public health service and insufficient knowledge about its harmful effects and costs. Although, screening exists in the US and some European countries, in Sweden the recommendation

has been to start with feasibility studies and to evaluate the results. Since 2008 a screening program for CRC has been implemented in Stockholm County [55, 56].

2. MOLECULAR GENETICS

Cancer (from the greek word *karkinos* meaning crab) is characterized by uncontrolled cell proliferation and by the capability of tumor cells to invade neighboring tissues and metastasize. There is nowadays wide acceptance that cancer development is a process of molecular events involving genetic or epigenetic changes that affect cell to cell signal transmission, cell cycle function, genome integrity and angiogenesis. Three types of genes are involved in the carcinogenic pathway: tumor suppressor genes, oncogenes and DNA repair genes.

Tumor suppressor genes are genes that exert an inhibitory function on cell proliferation. The products of these genes play an important role in cell cycle regulation, apoptosis control, suppression of growth factors and as negative regulators in signaling pathways. The main tumor suppressor genes involved in CRC tumorigenesis are *APC*, *DCC* and *TP53* [57]. Mutations in tumor suppressor genes usually have a recessive effect. Thus, according to the classical two-hit hypothesis of Knudson [58], both alleles need to be knocked out by a mutagenic event in order for the gene function to be lost. The first may be a somatic or germline mutation, while the second tends to be a partial or complete deletion of the other chromosome, so called loss of heterozygosity (LOH).

Proto-oncogenes/oncogenes are genes that by mutation become activated or hyperactivated, thereby promoting a carcinogenic development. The product of these genes, called oncogenes after activation, can affect functions such as response to growth factors by producing inappropriate stimulatory signals. The most important proto-oncogene in the tumorigenesis of CRC is *KRAS* [57]. Mutations in proto-oncogenes typically have a dominant effect, which means that only one of the two alleles needs to be mutated.

DNA repair genes are genes involved in preserving the integrity of the genome by correcting mistakes that occur during the DNA replication. At least seven mismatch repair (MMR) genes are known in humans, the most commonly involved in CRC development being *MLH1, MSH2, MSH6 and PMS2*. The proteins encoded by these genes function by recognizing and repairing single mismatched base pairs and nucleotide insertions or deletions. A germline mutation in MMR genes or epigenetic silencing by methylation of these genes will result in the accumulation of thousands of frameshift mutations in coding and non-coding repetitive DNA sequences (so called microsatellites)[59, 60].

The carcinogenesis of CRC is one of the most well-characterized pathways to malignancy in humans. Although the complexity of the molecular events behind this process has gradually been unraveled, the multistep model with sequential and additive genetic hits presented by Fearon and Vogelstein in 1990 [57] still holds up (Figure 5). Today, two major pathways to the development of CRC are established. However, other routes, such as the

serrated/CIMP pathway, have been discovered and cross-talk between the different pathways involved in CRC carcinogenesis has been suggested.

Chromosomal instability (CIN) pathway

This "canonical" pathway is believed to be responsible for 80-85% of all CRCs, including tumors in the FAP syndrome, and follows the model outlined by Fearon and Vogelstein. It is believed that the majority of CRCs arise from pre-existing adenomas and this model correlates the specific sequential genetic events to the evolving morphology in the adenoma-carcinoma sequence according to Figure 5. The most frequently observed chromosomal losses in CRC are seen in regions 5q, 17p and 18q which harbor the important tumor suppressor genes *APC*, *TP53* and *DCC*. Activation of *KRAS* is seen in about 50% of carcinomas and adenomas greater than 1 cm in size [61, 62.] Although the proposed order for genetic alterations in Figure 5 exists, the order of these events is not invariant. In fact, the accumulation of the multiple genetic hits in both oncogenes and tumor suppression genes seems to be most the important [57].

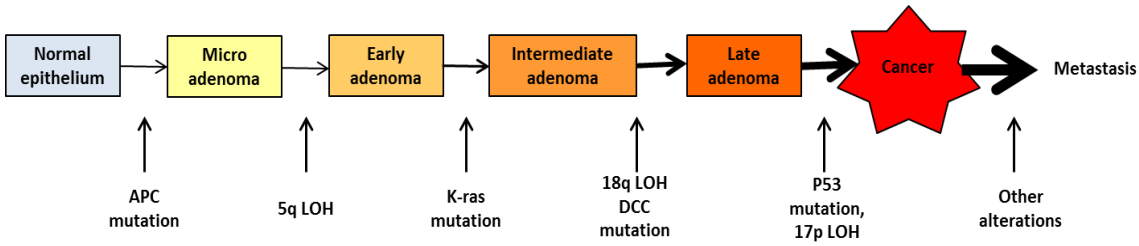


Figure 5. Molecular alterations in the chromosomal instability (CIN) pathway. Modified from Fearon & Vogelstein (1990) and Moran et al (2010).

Microsatellite instability (MSI) pathway

Microsatellites are short repetitive tandem sequences that are scattered through the human genome, both in coding and non-coding sequences. The MSI or mutator pathway, which is present in 12-20% of sporadic CRCs and in patients with LS, is characterized by a huge accumulation of mutations in these sequences, so called microsatellite instability (MSI) [60, 63]. This accumulation of frameshift mutations is caused by a primary defect in the MMR genes. The proteins encoded by these genes recognize mismatched bases in DNA during replication and are responsible for recruiting the helicase and exonucleases necessary for removal of the mismatch. When MMR proteins are functional, errors made by DNA polymerase in microsatellite sequences during replication is repaired. However, tumors with a high level of microsatellite instability are characterized by a 100-1000 fold higher mutation rate than in normal cells. The MMR genes most frequently associated with MSI CRCs are *MLH1* (mut L homolog 1, 3p21), *MSH2* (mut S homolog 2, 2p22), *MSH6* (mut S homolog 6, 2p16) and *PMS2* (postmeiotic segregation 2, 7p22)[64-67], while *MLH2*, *MLH3*, *MSH3*, *PMS1* and *Exo1* are believed to be involved to a lesser extent. The MMR proteins work in heterodimeric complexes when active in DNA repair (Figure 6) [68, 69]. There is data supporting the idea that loss of *MLH1* and *MSH2* is associated with

complete inactivation of MMR function, whereas defects in the other proteins only cause partial MMR deficiency [70].

MMR genes can be silenced either by a germline mutation plus a second hit (most often affecting *MLH1* or *MSH2*) as in LS, or by bi-allelic epigenetic silencing through hypermethylation of the promotor of *MLH1*, as in sporadic MSI tumors. Most sporadic MSI-H tumors show the CpG methylator phenotype (see below) characterized by widespread DNA hypermethylation [71]. Big cytogenetic abnormalities as in the CIN pathway are usually not detected in sporadic MSI-H tumors. Instead, mutations are seen in microsatellite sequences in genes associated with CRC, such as *TGFRβ2* (transforming growth factor beta receptor type 2), *IGF2R* (insulin-like growth factor receptor II), *BAX* (BCL2-associated protein X), *APC*, *β-catenin* and *MMP-3* (matrix metalloproteinase 3) [72-77]. MSI status of tumors can be determined by using PCR. According to international consensus criteria a panel of five microsatellite sequences is proposed for defining MSI. The recommended panel consists of two mononucleotide repeats and three dinucleotide repeats. Tumors with a high level of microsatellite instability (MSI-H) are defined as having instability in two or more markers, whereas tumors with low microsatellite instability (MSI-L) have instability in only one marker [78]. Microsatellite stable (MSS) tumors show no instability in any of the five loci. Instability is defined as a change in any length due to either insertion or deletion in repeating units in a microsatellite within a tumor, compared to normal tissue. An alternative to PCR based methods for MSI is immunohistochemical staining for each of the MMR proteins to detect loss of expression compared to normal tissue. This method is easy to perform and allows for pinpointing of the mutated gene.

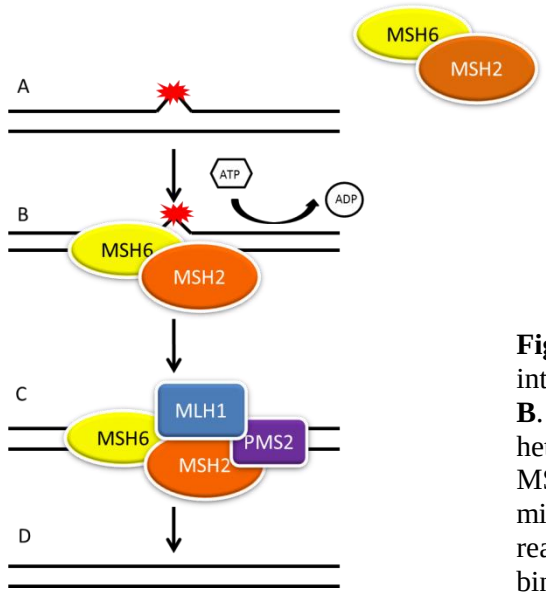


Figure 6. **A**. A mismatched nucleotide is introduced in DNA during a replication error. **B**. The mispaired base is recognized by a heterodimeric comlex of MSH2-MSH6 (or MSH2-MSH3). The complex binds to the mismatched base pair in an ATP dependent reaction. **C, D** A complex of MLH1-PMS2 binds to DNA and repairs the error.

The importance of recognizing MSI-H tumors lies in their distinct clinical and histopathological features. MSI-H tumors are located predominantly in the right colon and are reported to be more frequent in women [79, 80]. They also typically present with a greater depth of invasion but with a lower overall stage [79]. A better outcome for MSI-H tumors (whether sporadic or in LS) compared to MSI-L and MSS tumors has been reported by

many [81, 82]. The prognostic advantage of MSI-H seems to be most evident for stage II and stage III disease [82], but MSI status is considered to be a predictor of favorable outcome independent of stage [83]. MSI-H cancers display enhanced immunogenic properties which might contribute to the better outcome. The association between MSI-H and a good prognosis is independent of the mechanism behind it (germline mutation or silencing via hypermethylation). Interestingly, 5-fluorouracil based chemotherapy does not seem to provide a survival benefit among patients with MSI-H tumors, why this type of therapy should perhaps be avoided [82]. The histopathological profile of MSI-H tumors is dealt with in Chapter 4.

MSI-L cancers have been considered by some authors to be halfway between MSI-H and MSS. However, MSI-L tumors show clinicopathological and molecular characteristics more similar to MSS tumors with LOH and *KRAS* mutations [84], why they are usually grouped together with these.

Serrated/CIMP pathway

The characteristic histologic feature of polyps in the serrated group, hyperplastic, sessile serrated adenoma and traditional serrated adenoma, is the "saw-toothed" or stellate infolding of the crypt epithelium. Studies have shown that serrated polyps, especially sessile serrated adenomas, are more frequently associated to cancers that show MSI-H than to those that are MSS [85, 86]. The combination of a cytosine nucleotide followed by a guanine nucleotide (CpG dinucleotide) is uncommon in the human genome. However, dense clusters of CpG dinucleotides, named CpG islands, are found in the promotor region of half of all genes. Aberrant hypermethylation of these promoter islands, so called CpG island methylator phenotype (CIMP), has been associated with silencing of tumor suppressor genes and subsequent development of cancer [87]. In serrated adenomas with the MSI-H phenotype, such aberrant methylation of *MLH1* with loss of its expression is frequently noted. Also, in these tumors mutations of the same target genes as those in MSI-H cancers, for example *IGF2R, BAX and TGFβR2* have also been reported [73, 74, 88]. Further understanding of the serrated pathway has come from the discovery that mutations in the oncogene *BRAF* (v-raf murine sarcoma viral oncogene homolog B1) correlates with CIMP and occurs very early in the serrated pathway. There seems to be a synergistic effect of these two genetic events causing further progression of the lesion [89].

Genes related to invasion and metastasis

The capability of invasion and metastasis in CRC depends on a complex series of events including proteolysis of the local extracellular matrix, adhesion, angiogenesis, dissemination and cell growth. Several genetic alterations are involved in these processes. In the proteolysis step, proteinases such as the metalloproteinases (MMPs) degrade extracellular matrix components and enable cancer cells to detach from the primary tumor. MMP-7 (matrilysin) is overexpressed in the majority of CRCs and its expression is positively correlated with the metastatic potential of the tumor [90]. Many adhesion molecules including cadherins, integrins, VCAM-1 (vascular cell adhesion molecule 1)

and CEA (carcinoembryonic antigen) have been identified in CRCs. Cancer cells expressing these molecules are more likely to adhere to the extracellular matrix, leading to subsequent invasion and metastasis. However, downregulated expression of E-cadherin, a cell to cell adhesion molecule, is associated with invasiveness and metastatic potential of many cancers.

Angiogenesis is a crucial step in the progression of a tumor and provides a source for hematogenous dissemination and metastasis. Potential angiogenic factors include PD-ECGF (platelet-derived endothelial cell growth factor) and the six VEGF (vascular endothelial growth factor) molecules A-F. VEGF signal transduction involves binding to tyrosine kinas receptors, resulting in endothelial cell proliferation, migration, new vessel formation and increased vascular permeability. CRCs with increased VEGF expression are known to be associated with a poor prognosis [91].

3. PREDISPOSITION TO COLORECTAL CANCER

Twin studies have indicated that up to 35% of all CRCs can be ascribed to an inherited susceptibility [8]. The currently known high-risk syndromes such as FAP and LS however account for fewer than 5% of all CRC cases, leaving the majority with an unexplained genetic background. For individuals from unexplained family clusters with an affected first-degree relative, the lifetime risk of CRC is more than twice that of a general population [92]. Some of these cases may be the result of hitherto unexplained highly penetrant genetic changes, although most of the inherited susceptibility is believed to be the result of common low or moderate risk alleles that act in an additive or multiplicative way, or as modifiers of other risk factors. The approximate frequency of different types of CRCs in relation to the genetic background in a Swedish population is shown in Figure 7 [93, 94].

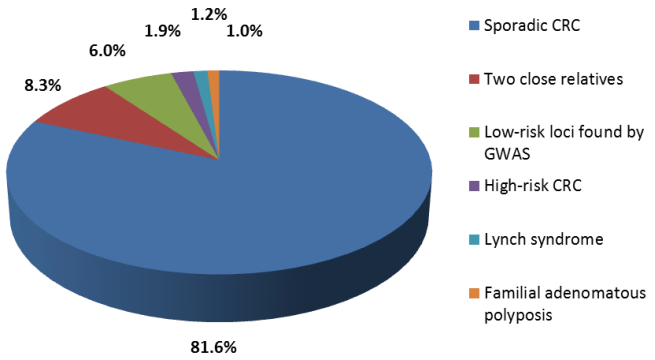


Figure 7. The genetic background of CRCs in a Swedish population. Modified from Picelli et al (2009) and Olsson and Lindblom (2003).

Colorectal cancer syndromes

Familial adenomatous polyposis (FAP)

FAP is an autosomal dominant syndrome characterized by the development of hundreds to thousands of adenomas throughout the colon and rectum, usually beginning in late childhood or adolescence. Because of the large number of polyps, several adenomas will inevitably develop into adenocarcinomas usually before the early forties. The penetrance of this disease is therefore 100% and the mean age at CRC diagnosis in untreated individuals is 40 years. The incidence of FAP is in the range 1: 30.000-7.000 and the syndrome accounts for less than 1% of all CRC cases. Apart from CRC, patients with FAP frequently develop small intestinal polyps, mainly duodenal adenomas, as well as gastric polyps, usually of the fundic gland type. The extra-gastrointestinal manifestations include a retroperitoneal or mesenterial fibromatosis called desmoid tumor (10-25% of patients), bone lesions such as exostoses and endostoses, dental abnormalities and epidermal cysts. Variants of FAP include Gardner's syndrome, Turcot syndrome and attenuated FAP (AFAP) [3].

A deleterious germline mutation in *APC* is seen in 95% of patients with classic FAP. In all individuals carrying this mutation, development of the syndrome follows the occurrence of a second hit which deletes the function of the remaining wild-type gene. 95% of the germline mutations are nonsense mutations due to insertions or deletions leading to an altered reading frame, producing a truncated protein [95]. The normal function of the APC protein as a negative regulator in the Wnt pathway is thereby disturbed leading to abnormal signal transduction and activation, as well as impaired cell adhesion (see Chapter 2).

Lynch syndrome (LS)

This syndrome, named after oncologist Henry Lynch, is an autosomal dominant disorder causing 1-3% of all CRCs. LS, previously called hereditary non-polyposis CRC (HNPCC), is the most common form of hereditary CRC. In contrast to FAP, patients with LS present with only a few polyps that within 1-2 years develop into cancer. Previously an average age at CRC diagnosis of 44 years has been reported, although recent population based data may suggest a later age of onset. The lifetime risk of developing CRC in LS depends on sex, type of gene involved and environmental risk factors and has been reported to be 69% for men and 52% for women. LS patients also carry an increased risk for cancer in other sites than the large bowel, including the endometrium (20-60% lifetime risk and the second most common cancer in LS), ovary, stomach, hepatobiliary tract, upper urinary tract, brain and skin. The combination of sebaceous gland tumors and LS-type internal malignancies is referred to as the Muir-Torre syndrome [3].

Before the discovery of MMR gene mutations as the cause of LS, clinical diagnostic criteria (Amsterdam I and II, see Table 1) [96, 97], where used to define families with this syndrome. However, in about half of the families that fulfilled these criteria neither MSI nor an MMR mutation could be found. Today the term LS is reserved for families with an identified pathogenic germline mutation in one of the four genes with a verified or putative function in MMR: *MLH1*, *MSH2*, *MSH6* and *PMS2* [98]. Deficiency in these genes will be manifested as MSI as discussed in Chapter 2. The Bethesda criteria (revised in 2002) [99] were created to select individuals that are suspected to have LS for MSI analysis (see Table 1).

Mutations, mostly truncating but sometimes missense, in *MLH1* and *MSH2* lie behind approximately 50% and 40% of LS cases respectively [100], while mutations in *MSH6* and *PMS2* are much more uncommon. *MSH2* mutations seem to confer a higher risk of extracolonic cancers than do *MLH1*, although there is no clear-cut correlation between the involved gene, mutation site or type, and the clinical picture. *MSH6* may however be associated with an elevated occurrence of endometrial carcinomas [101] and an "attenuated" type of LS caused by *MSH6* mutation and characterized by lower penetrance, has also been proposed [102]. MMR genes behave like tumors suppressors in that heterozygous cells can repair DNA normally. Thus, a second hit caused by deletion, mutation or methylation of the *MLH1* promoter in the wild-type allele is required for tumor development. CRCs in LS and the 10-15% of sporadic CRCs that are MSI-H positive display similar pathological features. Both show a predilection for the proximal colon (at least 60% of LS

cancers), although patients with sporadic MSI-H tumors tend to be older and lack a family history of CRC [103].

Table 1. Overview of Amsterdam I and II criteria for Lynch syndrome and revised Bethesda criteria.

Amsterdam criteria I There should be at least three relatives with CRC; all the following criteria should be present: 1. One should be a first-degree relative of the other two 2. At least two successive generations should be affected 3. At least one CRC should be diagnosed before the age of 50 years 4. Familial adenomatous polyposis should be excluded 5. Tumor should be verified by pathological examination
Amsterdam criteria II There should be at least three relatives with a Lynch syndrome-associated cancer (CRC, cancer of the endometrium, small bowel, ureter or renal pelvis); all of the following criteria should be present: 1. One should be a first-degree relative of the other two 2. At least two successive generations should be affected 3. At least one CRC should be diagnosed before the age of 50 years 4. Familial adenomatous polyposis should be excluded in the CRC case(s) if any 5. Tumors should be verified by pathological examination
Revised Bethesda criteria 1. CRC diagnosed in a patient less than 50 years of age 2. Presence of synchronous, metachronous colorectal, or other Lynch syndrome-related tumors* regardless of age 3. CRC with MSI-H phenotype** diagnosed at less than 60 years of age 4. Patient with CRC and a first-degree relative with a Lynch syndrome-related tumor, with one of the cancers diagnosed before the age of 50 years 5. Patient with CRC with two or more first- or second-degree relatives with a Lynch syndrome-related tumor, regardless of age
CRC, colorectal cancer * Lynch syndrome-related tumors include colorectal, endometrial, stomach, ovarian, pancreas, ureter, renal pelvis, biliary tract and brain-tumors, sebaceous gland adenomas, keratoacanthomas and carcinoma of the small bowel ** Tumor infiltrating lymphocytes (TILs), Crohn-like peritumoral lymphocytic reaction, mucinous/signet-ring differentiation or medullary growth pattern

Familial colorectal cancer type X

About half of families fulfilling the Amsterdam I criteria show no evidence of a heritable MMR defect, either by gene sequencing or tumor phenotyping for MSI. In addition, individuals in theses pedigrees display only a modest increase in the incidence of CRC and no increased risk of other types of LS-related cancers. The mean age of the patients in this Amsterdam I-positive MSI-negative group, coined familial colorectal cancer type X

(FCCTX), is also higher than in LS patients (60.7 versus 48.7 years) [104]. Also, in contrast to LS, tumors in FCCTX tend to be left-sided and show a slower adenoma-carcinoma progression rate [105]. Very little has been elucidated about the mechanisms behind this form of familial CRC. It has been suggested that this is a heterogenous group comprised of (1) some cancers aggregating by chance alone, (2) some aggregation related to shared lifestyle factors and (3) some yet to be defined genetic changes [104].

Other colorectal cancer syndromes and entities

MUTYH-associated polyposis, Peutz-Jeghers syndrome, juvenile polyposis syndrome, Cowden syndrome and hereditary mixed polyposis syndrome are all uncommon entities for which the genetics at least in part have been unraveled. There is, however, support for the hypothesis of additional high-risk monogenic syndromes for which the molecular background has not yet been defined. In a Swedish survey the frequency of non-FAP non-LS families having three or more first-degree relatives with CRC in at least two generations, i.e. showing a dominant pattern, was 1.9%. In addition, 8.3% of CRC cases came from families with two affected first- or second-degree relatives, where the risk for CRC is lower [93]. There is also evidence for rectal cancer as separately inherited entity [106] and a serrated polyposis syndrome (Jass syndrome) has been described [85, 86].

The search for low-risk genetic variants

Since the known high-risk syndromes only account for a small minority of CRC cases there has been an intensified search for low-penetrance genetic variations that probably underlie the major part of the hereditary disposition and together with environmental interactions are responsible for CRC as a complex disease.

Linkage analysis has been the classic method of choice for finding genes causing monogenic Mendelian diseases, such as in FAP and LS. In this method a number of DNA markers of known position are tested in family members segregating the disease. The closer two loci are on a chromosome the less likely they will be separated by recombination. By identification of DNA markers that co-segregate with the disease more often than expected by random segregation, the chromosomal region that harbors the responsible gene is located. The use of linkage analysis in the search for new syndromes in non-FAP non-LS families has yielded divergent results and loci associated to CRC have been suggested on chromosome 1, 3, 6, 7, 9, 11, 14, 15, 17 and 21. The loci on chromosome 3, 9 and 15 have been replicated in independent studies [107-109]. Linkage analysis however requires the use of large families and clearly defined genotypes. The method also has low power in detecting weak effects and high sensitivity to locus heterogeneity. Thus, when the penetrance of the disease is low the locus is usually difficult or impossible to identify by linkage since too many unaffected individuals who carry the allele will confound the calculations [110]. One possible way to minimize the problem with locus heterogeneity might be to subgroup the families according to differences in phenotype (such as tumor morphology) or according to the degree that they are affected.

Genome-wide association studies

In the past few years the search for novel susceptibility loci has been boosted by the emergence of genome-wide association studies (GWAS) and the use of single nucleotide polymorphism (SNPs). GWAS allows for the examination of genetic variants in a large population by comparing the frequency of an allele marker (usually a SNP) in a set of unrelated affected individuals (cases) with the frequency in a set of unaffected individuals (controls). Allelic association is present if the co-existence of a specific allele marker and the disease exceeds the expected occurrence based on random segregation. The term linkage disequilibrium is used to refer to allelic association between two linked loci. An association between the tested marker and the disease (phenotype) can result either from linkage disequilibrium between the marker and a closely located susceptibility gene or from a direct biological effect of the marker allele itself. The general rule of thumb is that the stronger the allelic association, the closer the marker is to the disease locus. Commonly used measures for association are the relative risk and odds ratio (OR) [110].

There are however problems with the use of association analysis in genomic scanning. First, there is the difficulty with multiple comparisons when so many tests are performed, because false-positive results are likely to occur by chance alone unless the usual significance levels (0.05 or 0.01) are modified. It is not clear what the appropriate correction should be since it depends on the underlying relationship between the markers, but typically the p-values must be very low (10^{-7} or 10^{-8}) to be considered significant in relation to the huge number markers (SNPs) that may be tested. Secondly, the association analysis rests solely on the assumption that some level of linkage equilibrium exists. Susceptibility alleles arising from frequent mutations or arising in genomic regions with very high recombination rates will have little or any detectable linkage disequilibrium. Thirdly, variables such as age, sex and the geographical or ethnical background of the population could potentially confound the results. Allelic association is population specific and special populations such as isolated or inbred populations can be especially useful in mapping complex traits [110]. The idea is that genetically isolated populations will have fewer genes contributing to a disease trait and therefore the effect of each remaining gene will be easier to detect. The advantage of the special population in its power to detect linkage however comes at the potential cost of specificity. If one or several susceptibility loci are detected, the effect of this gene or genes may be limited to the special population. However, many GWAS follow a setup where the first analysis in a discovery cohort is followed by validation of the most significant markers in an independent replication cohort [111-113].

SNPs

90% of all allelic differences existing within the human genome can be attributed to SNPs, which are nucleotide sequence variations in a single base pair between individuals or between the paired chromosomes. Usually SNPs have only two alleles and within a population SNPs can be assigned a major and minor allele frequency depending on which allele is the most or least frequent. The dbSNP database (www.ncbi.nlm.nih.gov/SNP/index.html) currently contains 10.4 million human SNPs which have been condensed into a non-redundant set of 4.8 million validated SNPs, yielding a SNP density

of 1 per 1.3 kb [110]. SNPs localized within a coding region have the greatest potential to affect the structure and function of a gene. Less than half of SNPs localized to such regions result in no change in the amino acid sequence because of codon redundancy (synonymous change), while the rest result in an amino acid alteration (non-synonymous change). Most SNPs are however located in non-coding regions such as introns, flanking sequences and splice sites, although effects on splicing, folding of mRNA and promoter function of these "non-coding" SNPs have been described [110]. Many different platforms have been developed for SNP analysis based on four basic allele-specific assays: (1) hybridization with allele-specific probes, (2) oligonucleotide ligation, (3) single-nucleotide primer extension and (4) enzymatic cleavage. Many of these techniques have been automated in commercial systems, including colorimetric microtiter-plate-based assays and microarray chips.

SNPs and colorectal cancer

The implementation of GWAS performed with SNP chips has led to the discovery of several susceptibility loci for CRC, some of which have been replicated in independent studies. A list of SNPs found, their locus and associated gene (if detected) is presented in Table 2. Most of these detected SNP variants confer an OR for CRC in the range 0.8 (some exert a protective effect) to 1.4 and are believed to be responsible for about 6% of the excess familial risk [113].

The first locus identified was 8q24 where the most significant SNP rs6983267 has been replicated in several studies [111, 114, 115]. This SNP maps close to the oncogene *MYC*, which is regulated by the Wnt-signaling pathway. Recently a study has reported that the risk genotype (GG) at this SNP affects the binding site for TCF4 (transcription factor 4) so that the transcription of *MYC* is upregulated [116]. Another locus is 18q.21.1 where the SNP rs4939827 maps to *SMAD7*, an intracellular antagonist of TGFβ signaling [112, 117]. The SNP rs3802842 on 11q23 is located close to a gene called *POU2AF1* which encodes a transcription factor. This SNP shows substantial population-specific differences in CRC risk. Both rs4939827 and rs3802842 show a higher risk for rectal cancer than for colon cancer [117]. The locus 15q13.3-q14, previously linked to hereditary mixed polyposis syndrome in individuals of Ashkenazi Jewish descent, might also harbor a low-risk variant that affects the *GREM1* (gremlin 1) gene which also involved in the TGFβ pathway [118, 119]. A meta-analysis of GWAS has identified 14q22.2 as a risk locus where the SNP rs 4444235 maps close to the transcription start site of the gene *BMP4* encoding bone morphogenic protein 4 [113]. BMP signaling inhibits intestinal stem cell self-renewal through suppression of the Wntβ-catenin signaling. The SNPs rs 10411210 and rs7259371 contain the *RHPN2* (Rho GTPase binding protein 2) gene involved in the regulation of actin cytoskeleton and cell motility [113, 120] and rs9929218 maps to the *CDH1* (cadherin 1) gene affecting the β-catenin T-cell transcription factor pathway [113, 121]. On 8q23 there is no certain disease causing gene, but the SNP rs16892766 is in linkage disequilibrium with a region that includes *EIF3H*, a gene involved in cell-growth and viability [122]. There are no evident protein-coding sequences in the vicinity of rs10795668 on 10p14 [122]. The same is true for the SNPs rs961253 & rs355527 on 20p12.3, although the *BMP2*-gene is located 342 kb telomeric to this site [113]. rs 7197259 on 9p24 is not

located within any gene. However, there are four genes nearby, none of which have been implicated in CRC so far [111, 123]. In a replication study of all of the above mentioned SNPs in Swedish cohort within the Swedish Low-Risk Colorectal Cancer study (see Chapter 7), five showed statistically significant ORs similar to previous reports: the SNPs on 8q23.3, 8q24.21, 10p14, 15q13.3 and 18q21.1. The loci on 11q23, 16q22.1, 19q13.1 and 20p12.3 showed weak trends towards association, but 9p24 and 14q22.2 were not confirmed. In addition, four correlations between SNPs and phenotypes were found: the G allele of rs6983267 showed an association to older age, the G allele of rs1075668 to younger age and sporadic cases, and the T allele of rs10411210 to younger age [124].

Table 2. CRC loci identified by genome-wide association studies (GWAS).

SNP ID	Locus	Gene
rs6983267	8q24.21	*MYC*?
rs16892766	8q23.3	?
rs10795668	10p14	?
rs4939827	18q21.1	*SMAD7*
rs3802842	11q23.1	*POU2AF1*?
rs4779584 & rs10318	15q13.3	*GREM1*
rs961253 & rs355527	20p12.3	?
rs4444235	14q22.2	*BMP4*
rs10411210 & 7259371	19q13.1	*RHPN2*
rs9929218	16q22.1	*CDH1*
rs719725	9p24.1	*?*

4. PATHOLOGY

CRC is a malignant tumor originating in the epithelium of the colon or rectum. More than 90% of CRCs are adenocarcinomas which usually develop from the precursor lesion adenoma. The definition of carcinoma in colon and rectum (unlike in the rest of the gastrointestinal tract) requires invasion through the lamina muscularis mucosae into the submucosa. Although lymphatic vessels are present in the colorectal mucosa metastatic spread is not believed to occur unless the muscularis mucosae is breached [3].

Macroscopic features

CRCs can grow in a polypoid (exophytic) fashion into the lumen or, more commonly, as an ulcerative (endophytic) lesion infiltrating into the wall (Figure 8). Annular growth with circumferential involvement and stenosis of the lumen is also common but diffusely infiltrative growth resembling linitis plastica of the stomach is rarely seen. Although there is significant overlap of features, carcinomas proximal to the splenic flexure tend to grow as exophytic masses while those distally in colon and rectum usually are more endophytic and annular. Most CRCs are homogenous and grey-white on the cut surface, often with necrosis, although mucinous tumors may be gelatinous. Sometimes penetration or napping of the serosal surface or overgrowth on adjacent organs may be detected macroscopically [3].

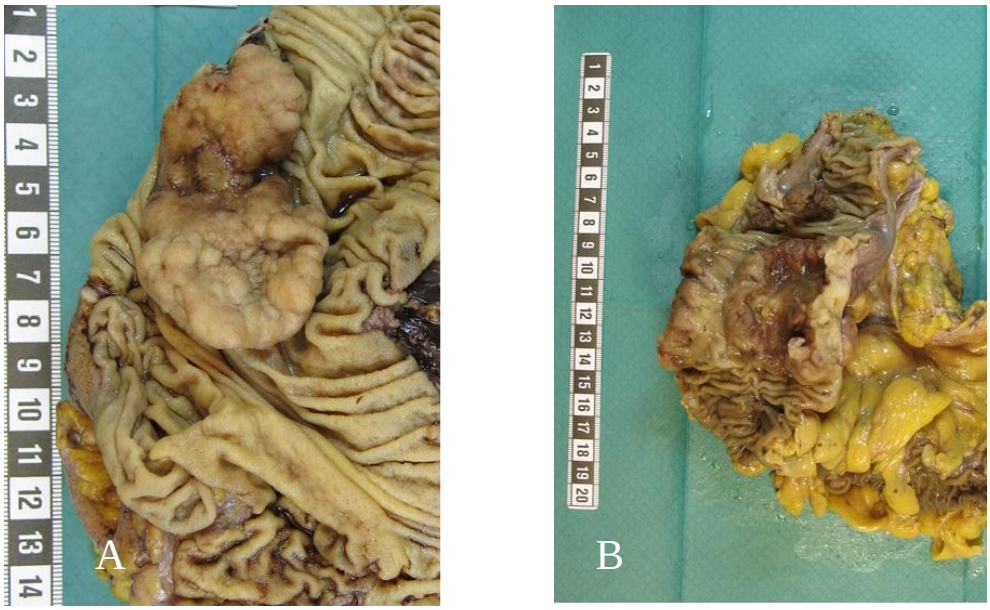


Figure 8. Colonic carcinomas after formalin fixation. **A**. Polypoid tumor of the hepatic flexure. **B**. Ulcerated tumor of the sigmoid covering a large part of the circumference.

Microscopic features

The majority of CRCs are typical adenocarcinomas composed of moderate to large sized irregular glands often containing necrotic debris in the lumen. The tumor cells are usually clearly atypical although still cylindrical and somewhat resembling the normal colonic mucosal cells. Often there is ulceration as well as some degree of desmoplastic stromal reaction and inflammatory response around the tumor. Perineural, lymphatic and venous invasion is not uncommon. At the periphery of the tumor sometimes a remnant of a pre-existing adenoma may be found. As stated above, the diagnosis of CRC requires invasion through the lamina muscularis mucosae. For lesions confined to the mucosa the term intramucosal carcinoma has been applied although this is equivalent to high-grade dysplasia.

Grading

Traditionally CRCs have been graded as well-, moderately or poorly differentiated on the basis of glandular formation according to Table 3. This classification is still the one widely used among Swedish pathologists. Recently a two-tiered grading system with only low-grade and high-grade has been proposed by the WHO, because of greater reproducibility and the similar clinical behavior of well- and moderately differentiated carcinomas [3]. Undifferentiated carcinoma (grade 4) is a term of exclusion reserved for carcinomas that show no morphological or immunohistochemical evidence of glandular formation, mucin production, or neuroendocrine, squamous or sarcomatoid differentiation. Grading is based on the least differentiated component of tumor, disregarding the deep invading front.

Table 3. Criteria for histological grading of colorectal adenocarcinomas (modified after WHO, 2010).

Criterion	Differentiation	Numerical grade*	Descriptive grade
>95% gland formation	Well-differentiated	1	Low
50-95% gland formation	Moderately differentiated	2	Low
0-49% gland formation	Poorly differentiated	3	High
*The category ”undifferentiated carcinoma” (grade 4) is reserved for carcinomas with no gland formation, mucin production or neuroendocrine, squamous or sarcomatoid differentiation.			

Specific features in CRC

Crohn-like peritumoral lymphocytic reaction is defined as the presence of nodular aggregates of mainly B-lymphocytes deep to the advancing tumor margin, usually located in the lamina muscularis propria or in the pericolic fibroadipose tissue. This reaction represents a host immune response towards the tumor and has derived its name from the resemblance to transmural lymphocytic aggregates, a hallmark of Crohn’s disease. The presence of Crohn-like reaction has been linked to improved patient survival in some

studies [125,126] and is one of the characteristics of MSI-H tumors. At least three nodular aggregates of lymphocytes within a single x4 field deep to the advancing tumor margin has been used as a definition of this feature [126].

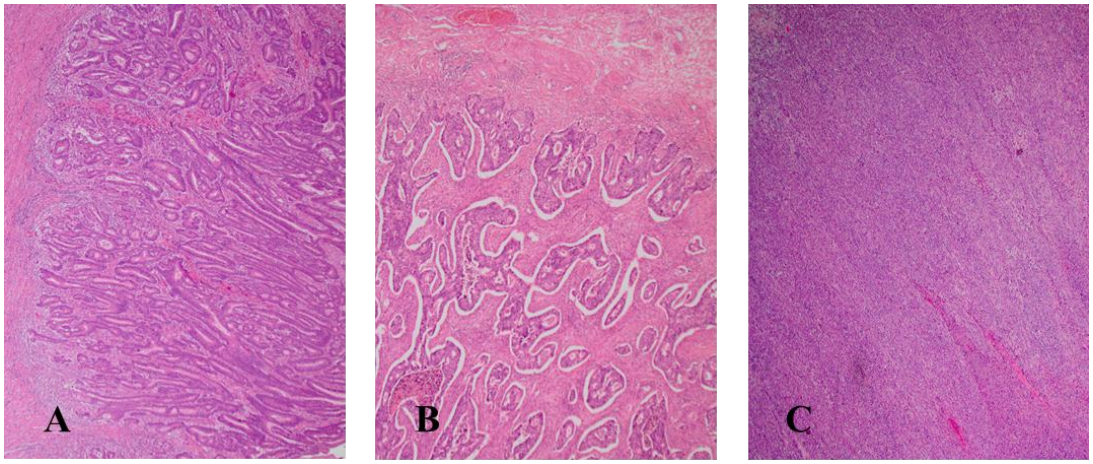


Figure 9. CRCs of different grades. **A**. Well-differentiated **B**. Moderately differentiated **C**. Poorly differentiated. Hematoxylin & eosin (H &E) staining, x40.

Tumor infiltrating lymphocytes (TILs) are intraepithelial, mainly cytotoxic, T-lymphocytes that are found within the tumor tissue. An abundance of TILs have been associated with improved clinical outcome [127, 128] and TILs are one of the most sensitive and specific features in predicting MSI-H [129]. The exact mechanism of TIL accumulation and its association to improved outcome has not been elucidated, although the adaptive immune system may play a role in suppressing tumor progression. TILs may reflect specific molecular alterations associated with indolent tumor behavior and it has been suggested that truncated peptides produced by frameshift mutations due to MSI may be immunogenic and contribute to the host immune response. It has also been proposed that MSI-H CRCs are less able to express functional Fas ligand and thereby less successful in killing lymphoid cells by Fas mediated apoptosis [129]. Several definitions of a high level of TILs have been used such as a cut-off value of 0.7, 2 or >3 TILs per high power field, or ≥5 TILs/100 cancer cells.

Desmoplasia, i. e. a hypocellular intense fibrous reaction around infiltrating tumor tissue, is often seen in CRC. There are conflicting reports regarding the role of stromal response in cancer development. It has been argued that it limits tumor aggressiveness and could represent an attempt by the host to seal off the tumor, which is also supported by some studies that show a survival benefit in cases with desmoplasia [130]. However, a fibrotic response could also favor the tumor by neovascularization and preventing access to host lymphocytes, macrophages and other immune regulatory cells. Focus has also been drawn not only to the amount of fibrosis, but also to its qualitative nature. In a study by Ueno et al, an immature fibrous stroma consisting of randomly oriented keloid-like collagen bundles in a myxoid tissue was a negative prognostic factor, as opposed to a denser mature collagen stroma [131].

Dirty necrosis or garland necrosis is the presence of large amounts of cell detritus and inflammatory cells within the glandular lumina. It is often considered a characteristic of CRC. The absence of this feature has however been described as a marker for MSI-H tumors, especially if it is combined with mucinous differentiation and a high number of TILs [79, 132].

Vascular invasion, both venous and lymphatic, has been found to be an independent prognostic factor in both univariate and multivariate analyses [91, 133-135]. In some studies the location of vascular invasion in extramural veins has been of prognostic value [136]. The diagnosis of intravascular tumor growth is often difficult to make because fixational artefacts with retraction of tumor strands in fibrotic tissue can mimic vascular invasion. The frequency of vascular invasion is reported to vary from 10 to 89.5% [137], with false-negative rates between 10.5 and 29.6% if only hematoxylin & eosin (H & E) staining is used [138]. The frequency is also influenced by the number of blocks taken and if tangential sectioning is performed. The assessment of vascular invasion can be improved with immunohistochemical staining for endothelial markers such as CD31 or CD34, and lymphatic spaces can be differentiated from venous by their positivity for the immunomarker D2-40.

Perineural invasion is defined as tumor cells infiltrating underneath the perineurium at the invasive margin of the tumor or deep to it. In a number of multivariate studies this feature has been shown to be an independent indicator of poor prognosis [139].

Budding is defined as the detachment of single isolated cancer cells or a cluster of up to four cells in the stroma at the invading front of the tumor. This feature, which represents dedifferentiation of the tumor and the first step of invasion and metastasis, has been shown to be an independent adverse prognostic factor [140]. Attempts to quantify budding have been made and immunohistochemical staining for cytokeratins can be used to highlight this feature.

Tumor margin configuration has been reported to have prognostic significance that is independent of stage. An infiltrative irregular pattern of growth is an adverse prognostic factor as opposed to a circumscribed smooth-pushing pattern [139, 141]. However, interobserver variability among pathologists in evaluating this feature is high with only fair agreement as to what should be called an infiltrating growth pattern [142].

Immunohistochemistry

Most CRCs are negative for cytokeratin 7 (CK7) but positive for cytokeratin 20 (CK20). However 10% of CRCs are extensively positive for CK7 and approximately 5% are negative for CK20. CK7 staining is increased and CK20 staining is decreased in MSI-H tumors. CDX2 (caudal-type homeobox protein 2) stains 98-100% of all CRCs. Expression of CDX2 is not associated with MSI status. In addition, CRCs are usually positive for CK8, CK18, CK19 (low molecular weight cytokeratins) and 40% stain for MUC2 (intestinal type of mucin) [143, 144].

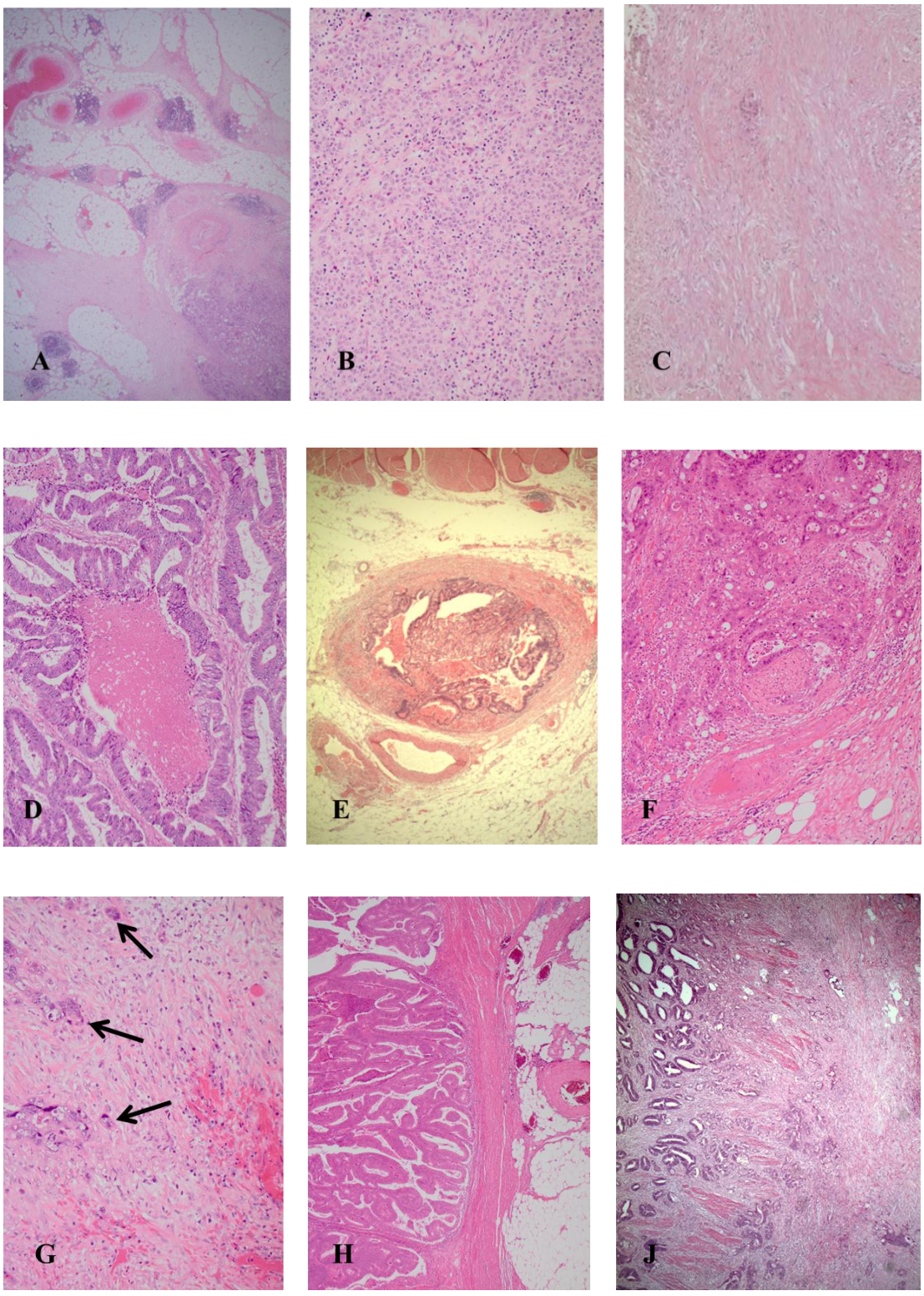


Figure 10. Specific features of CRCs: **A**. Crohn-like peritumoral lymphocytic reaction (x20). **B**. Tumor infiltrating lymphocytes (TILs) in a poorly differentiated tumor with medullary features (x200). **C**. Desmoplasia (x100). **D**. Dirty necrosis (x100). **E**. Vascular (venous) invasion (x25). **F**. Perineural invasion (x100). **G**. Budding (x200). **H**. Circumscribed tumor margin (x40). **J**. Infiltrative tumor margin. (x25). H & E staining.

Special variants of CRC

Mucinous adenocarcinoma

This type of CRC is composed to >50% of pools of extracellular mucin that contain malignant epithelium in the form of acinar structures, strips of tumor cells or individual tumor cells (Figure 11A). Signet-ring cells may be seen. 10-20% of CRCs are described as mucinous and these tumors have poorer 5-year survival compared to non-mucinous CRCs [145], although results are conflicting [146]. According to WHO (2010) the differentiation of a mucinous cancer is determined by the level of maturation of the malignant epithelial cells, but according to Swedish consensus criteria and older WHO criteria (2000) mucinous cancers and signet-ring cell cancers have by definition been classified as poorly differentiated. Many mucinous carcinomas are however MSI-H positive and thereby low-grade [147]. Carcinomas with <50% mucinous areas are categorized as having a mucinous component [3].

Signet-ring cell carcinoma

This type of CRC is sometimes considered a subtype of mucinous carcinoma and is defined by >50% tumors cells with a prominent intracytoplasmatic vacuole and typically displacement of the nucleus, so called signet-ring cells (Figure 11B). These cells can occur floating in pools of free mucin or infiltrating in a diffuse manner within a fibrous stroma (linitis plastica-pattern). Carcinomas of the signet-ring cell type comprise only 0.7-2.6% of all CRCs. Compared to both conventional adenocarcinomas and mucinous adenocarcinomas without signet-ring cells, they tend to present at a higher T-stage and with a higher number of lymph node metastases. They also show a poorer outcome with a higher rate of distant recurrence and decreased survival [148]. Some signet-ring cancers are however MSI-H positive and thereby low-grade. Signet-ring cell carcinomas develop through a separate genetic pathway showing disruption of the E-cadherin/β-catenin complex involved in cell to cell adhesion. A different pattern of alterations from conventional colorectal adenocarcinomas has also been shown in growth kinase-related oncogenes (*KRAS, BRAF*), tumor suppressor genes (*TP53, TP16*), gene methylation and COX-2-expression [148, 149].

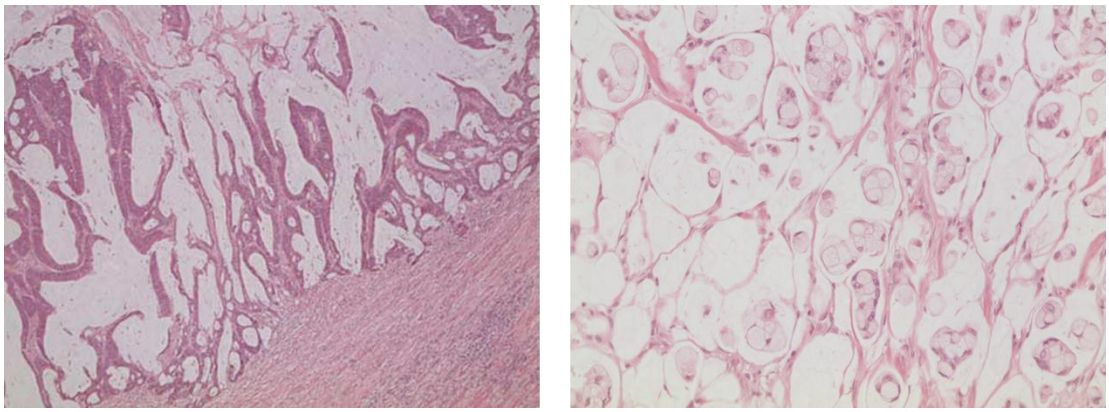


Figure 11. A. Mucinous adenocarcinoma (x25). **B** Signet-ring cell carcinoma (x200) H & E staining.

Medullary carcinoma

This rare tumor (0.03% of all surgically removed CRCs) is made up of sheets of undifferentiated epithelial cells with vesicular nuclei, prominent nucleoli, abundant pink cytoplasm and a conspicuous element of TILs. Although morphologically similar to poorly differentiated adenocarcinomas these tumors display a distinct clinical behavior. They are more common in older women, more common in right than in left colon, less likely to show lymph node metastases and generally carry a better prognosis. Medullary carcinomas are associated to MSI-H in most cases [150].

Other rare variants

Serrated adenocarcinomas are architecturally similar to sessile serrated polyps with stellate or saw-tooth glands. These tumors can be MSI positive or show *BRAF* mutations and CpG island hypermethylation. Adenocarcinomas with neuroendocrine differentiation occur, as well as pure neuroendocrine tumors and carcinomas. Cribriform comedo-type adenocarcinoma, micropapillary adenocarcinoma, adenosquamous carcinoma and spindle cell carcinoma are unusual variants. Undifferentiated carcinoma is described above [3].

Morphology of MSI-H positive tumors

Since the beginning of the 1990s when MMR-deficient tumors and MSI were described it has been recognized that these tumors show a distinct phenotype. Clinicopathological findings that have been associated with MSI-H positive CRCs (either sporadic or in LS) are proximal anatomical location, multiple cancers, poor differentiation (including medullary type), mucinous differentiation (including signet-ring cell carcinoma), histologic heterogeneity (i.e. at least two distinct growth patterns), Crohn-like peritumoral lymphocytic reaction, TILs, absence of dirty necrosis and circumscribed tumor margin [79, 103]. Several reports however point out TILs as the best morphological biomarker of MSI-H tumors [79, 80, 129]. In one study a cut-off of >2 TILs per high-power field resulted in 90% sensitivity and 77% sensitivity for MSI-H. The sensitivity was increased to 100% by the addition of two other features: any amount of mucinous differentiation and the absence of dirty necrosis [79]. The Bethesda criteria (revised in 2002), which are designed to identify individuals at risk for LS, recommend MSI testing of tumors showing TILs, Crohn-like peritumoral lymphocytic reaction, mucinous/signet-ring differentiation or medullary growth pattern in individuals less than 60 years [99].

Immunohistochemistry of MSI-H tumors

Immunohistochemistry for MMR proteins shows good correlation to PCR for MSI why this method is nowadays widely used in the detection of MMR defect tumors. Staining for the most commonly affected MMR proteins MLH1, MSH2, MSH6 and PMS2 will show lack of staining in tumor nuclei compared to normal tissue in the case of an MMR-deficient CRC. Studies have shown both high sensitivity (92-92.3%) and specificity (99.8-100%) for immunohistochemistry [151, 152]. The advantage of immuno-histochemistry over PCR-MSI is that it can pinpoint the mutated gene, although there is a risk of missing a small proportion (8%) of MSI-H tumors that show normal expression of a protein which, however, is non-functional due to truncating or missense mutations [152].

Staging

CRCs can progress with local invasion or show lymphatic or hematogenous spread. Colonic carcinomas may after growing through lamina muscularis propria extend directly to the serosal surface with peritoneal carcinomatosis. Perforation can occur and the tumor may become adherent to adjacent structures or infiltrate directly into adjoining organs. Advanced rectal cancers can infiltrate into pelvic structures such as the vagina or urinary bladder [3]. Originally it was believed that CRCs follow an orderly progression from local tumor invasion to subsequent lymphatic or hematogenous spread after penetrating the intestinal wall. However, today it is known that some tumors show lymph node metastases or develop distant disease although they have not penetrated the bowel wall. The liver is the most common site for hematogenous spread of CRC, occurring in in about 50% of cases, and the lung is the second most common. Tumor spread to other sites in the absence of lung or liver metastases is uncommon [145].

All staging systems for CRC, including the original classification for rectal cancer by Cuthbert Dukes as well as the modified by Astler-Coller, are based on the extent of tumor spread through the wall and the presence of lymph node or distant metastases. The systems mentioned above are now replaced by the TNM classification [153] which forms the base for the staging system proposed by American Joint Committee on Cancer and the International Union Against Cancer (AJCC/UICC¸ see Tables 4 and 5). In addition to the TNM variables there are optional descriptors L, V and Pn for lymphatic, venous and perineural invasion. The prefix p in pTNM is used to indicate pathological, as opposed to clinical or radiological, assessment. The prefix y as in ypTNM signals that the classification is performed during or following multimodality therapy such as preoperative radiochemotherapy. In Sweden the optional subclassification of T3 tumors into a through d is used (see Table 6).

In general, all lymph nodes in a surgical specimen of CRC are sampled by the pathologist. However it has been shown that at least 12 to 15 lymph nodes must be examined to accurately predict regional lymph node negativity (N0) [154]. For this reason it has been postulated that 12 lymph nodes be considered the minimum acceptable harvest.

Prognostic factors and features

Stage, i.e. the pTNM classification, is the strongest prognostic factor for CRC. However, features with adverse effect on outcome include bowel obstruction or perforation, extensive circumferential tumor involvement, poor differentiation and signet-ring cell carcinomas (with exception for MSI-H tumors), infiltrative tumor margin, budding and invasion in lymphatic, venous or perineural spaces. A short distance between the resection margin and tumor and incomplete excision with residual tumor also carry an adverse prognosis [3]. CRM involvement in rectal cancer may be the single most critical pathological factor in predicting local recurrence [155] and has also been shown to predict

distant recurrence and overall survival [139]. Size is of no prognostic significance in CRC [137]. Features with positive effect on outcome are signs of favorable host response such as TILs and Crohn-like peritumoral lymphocytic reaction as well as reactive lymph nodes.

Several potential molecular or immunohistochemical prognostic or predictive markers have been described in the literature but none has yet been introduced in routine practice. MSI-H has however proved to be a sign of favorable outcome (hazard ratio about 0.65) according to previous discussion. Among other proposed biomarkers are 18q LOH/DCC and mutation of *KRAS* and *BRAF* [156]. Recently the immunohistochemical expression of ezrin, a molecule involved in plasma membrane stabilizing as well as membrane receptor function, has been reported to predict time to local recurrence in rectal cancer [157].

Table 4. TNM (7th edition) classification for carcinomas in colon and rectum.

	Primary Tumor
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis[1]	Carcinoma in situ: intraepithelial or invasion of lamina propria
T1	Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades subserosa or into non-peritonealized pericolic or perirectal tissues
T4	Tumor directly invades other organs or structures and/or perforates visceral peritoneum **T4a** Tumor perforates visceral peritoneum **T4b** Tumor directly invades other organs or structures [2, 3]
Notes:	1. Tis includes cancer cells confined within the glandular basement membrane (intraepithelial) or mucosal lamina propria (intramucosal) with no extension through the muscularis mucosae into the submucosa. 2. Direct invasion in T4b includes invasion of other organs or segments of the colorectum by way of the serosa, as confirmed on microscopic examination, or for tumors in a retroperitoneal or subperitoneal location, direct invasion of other organs or structures by virtue of extension beyond the muscularis propria. 3. Tumor that is adherent to other organs or structures, macroscopically, is classified cT4b. However, if no tumor is present in the adhesion, microscopically, the classification should be pT1-3, depending on the anatomical depth of wall invasion.

N	Regional Lymph Nodes
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1-3 regional lymph nodes
	N1a Metastasis in 1 regional lymph node **N1b** Metastasis in 2-3 regional lymph nodes **N1c** Tumor deposit(s), i.e. satellites*, in the submucosa, or in non-peritonealized pericolic or perirectal soft tissue *without* regional lymph node metastasis
N2	Metastasis in 4 or more regional lymph nodes
	N2a Metastasis in 4-6 regional lymph nodes **N2b** Metastasis in 7 or more regional lymph Nodes
Note:	* Tumor deposits (satellites), i.e. macroscopic or microscopic nests or nodules, in the pericolorectal adipose tissue's lymph drainage area of a primary carcinoma without histological evidence of residual lymph node in the nodule, may represent discontinuous spread, venous invasion with extravascular spread (V1/V2) or a totally replaced lymph node (N1/N2). If such deposits are observed with lesions that would otherwise be classified as T1 or T2, then the T classification is not changed, but the nodule(s) is recorded as N1c. If a nodule is considered by the pathologist to be a totally replaced lymph node (generally having a smooth contour), it should be recorded as a lymph node and not as a satellite, and each nodule should be counted separately as a lymph node in the final pN determination.

M	Distant Metastasis
M0	No distant metastasis
M1	Distant metastasis
	M1a Metastasis confined to one organ (liver, lung, ovary, non-regional lymph node(s)) **M1b** Metastasis in more than one organ or the peritoneum

Table 5. Staging of colon and rectal cancer (TNM, 7th edition).

Stage 0	Tis	N0	M0
Stage I	T1, T2	N0	M0
Stage II	T3, T4	N0	M0
Stage IIA	T3	N0	M0
Stage IIB	T4a	N0	M0
Stage IIC	T4b	N0	M0
Stage III	Any T	N1, N2	M0
Stage IIIA	T1, T2 T1	N1 N2a	M0 M0
Stage IIIB	T3, T4a T2, T3 T1, T2	N1 N2a N2b	M0 M0 M0
Stage IIIC	T4a T3, T4a T4b	N2a N2b N1, N2	M0 M0 M0
Stage IVA	Any T	Any N	M1a
Stage IVB	Any T	Any N	M1b

Table 6. Optional subclassification of T3 tumors.

T3	Tumor invades subserosa or into non-peritonealized pericolic or perirectal tissues
T3a	Invasion < 1mm into subserosa or non-peritonealized pericolic or perirectal tissues
T3b	Invasion 1-5 mm into subserosa or non-peritonealized pericolic or perirectal tissues
T3c	Invasion 5-15 mm into subserosa or non-peritonealized pericolic or perirectal tissues
T3d	Invasion > 15 mm into subserosa or non-peritonealized pericolic or perirectal tissues

5.CRC IN RELATION TO PATIENT CHARACTERISTICS

CRC and sex

Studies of CRC have shown female patients to be older and to have more proximal and poorly differentiated tumors than males [158, 159], as well as more MSI-H tumors [160]. Two retrospective analyses have also reported more advanced stages of cancer in women compared to men [158, 161]. The majority of studies that have assessed sex and overall survival have reported no significant associations [162]. However in one study, women aged 50 years and above had poorer cancer-specific survival than men independent of age, emergency surgery, site, grade and stage, while young women (below 50 years) had a significantly better overall survival compared to young men [159]. The survival advantage of young premenopausal women has been proposed to be due to the protection conferred by estrogen, which is lost in postmenopausal women [159].

There is clinical evidence that estrogen protects against the development of CRC. Hormone replacement therapy reduces CRC mortality and parity has been inversely associated to the rate of CRC [160]. The way by which estrogen prevents the development of CRC is complex and has not been fully elucidated, although different mechanisms have been proposed.

CRC and age

Approximately 8 % of all CRCs occur in persons younger than 50 years and 2-3 % in persons younger than 40 years [163]. Studies of the clinicopathological profile of CRC in relation to age have shown contradictory results. According to some studies patients younger than 50 years present with less localized and more distant disease (i.e. higher stage), as well as a higher rate of poorly differentiated tumors [163]. There is no definite explanation for this, but it is possible that that young patients present with later disease because they are not screened or because of delay in patient presentation or lack of awareness of the disease, both among patients and physicians. They may also be at higher risk because of a higher prevalence of conditions predisposing them to CRC such as a family history of the disease. However, one cannot rule out that young patients present at a higher stage because of tumors that per se, because of genetic or other biological reasons, are more aggressive. On the other hand, some studies have shown that stage at presentation and survival figures for young patients are comparable to those reported in older age groups [164].

Mucinous tumors have been described to be up to four times more frequent in young patients compared to elderly, comprising 20 % of all CRCs in the young group. This type of tumor in the young has been associated with an increased risk of local recurrence [164]. A high number of lymph node metastases, vessel invasion, and infiltrating tumor margin are

reported to be more common among patients below 50 years. These findings are also in line with a more aggressive histopathological profile. In addition, both young men and old women show a relatively high frequency of right-sided tumors [165].

CRC and location

The right side of colon is usually defined as the portion including caecum, ascending colon, the hepatic flexure and transverse colon, while the left side is defined as the distal portion from the splenic flexure, i. e. descending colon, the sigmoid and rectum. In some studies the splenic flexure is included in the right colon.

When comparing CRCs in different locations, right-sided lesions in general show more aggressive features than left-sided as reflected in morphology and stage. Poor differentiation, mucinous type, larger size, higher TNM-stage, vessel invasion and expanding tumor margin occur more frequently in right-sided lesions, while annular and polypoid growth and an infiltrating tumor margin are more common in left-sided lesions [165]. Conversely, poorly differentiated and mucinous tumors are more frequently seen in the right colon [166]. Right-sided colon cancers also show a higher frequency of node positive disease as well as a shorter median survival compared to left-sided (78 vs. 89 months, $p<0.001$) [167]. In accordance to above, there is a gradual increase in the ratio of right to left colon cancer with age in female patients. In male patients, there is a greater proportion of left-sided cancers in middle-aged, while right-sided lesions predominate in young and old age groups [165].

Since the 1980s there has been a persistent increase in the percentage of right-sided colon cancers with an associated decrease in the percentage of left-sided colon and rectal cancers [167, 168]. The cause behind this is poorly understood and likely multifactorial. It may reflect the growing use of colonoscopy and screening, as well as a changing age and sex distribution of the disease since elderly patients and women tend to have more right-sided tumors. Changing dietary habits (high fat and low fiber) has also been implied. The left-to-right shift of incidence is reported to be higher among women than men [169].

CRC and family history

The clinicopathological characteristics of LS, FAP and other CRC syndromes are well known. However, the morphological profile of the majority of familial CRC cases is unknown. Patients with a family history of CRC have been shown to be relatively younger and more likely to carry right-sided tumors. Also, sigmoidal and rectal cancers appear to be less frequent in patients with a positive family history of CRC compared to sporadic cases [93, 170]. Few studies have addressed the histopathological profile of non-LS non-FAP familial CRCs, although there are comparisons of the morphology of tumors in LS and FCCTX. These reports have shown that cancers in FCCTX more often are located in the distal colon and rectum, more often show lymph node metastases and usually display conventional glandular morphology in contrast to the medullary or signet-ring cell features of LS tumors. Also, findings associated with LS such as poor differentiation,

mucin production, TILs, Crohn-like peritumoral lymphocytic reaction, lack of dirty necrosis and circumscribed tumor border, are less often found in FCCTX. In addition, patients with FCCTX have a lower risk of CRC, develop tumors at a later age, display more aneuploidy tumors and have less often extracolonic tumors in their families compared to patients with LS [171, 172, 105]. Although these morphological and clinical finding support the existence of FCCTX as a separate entity from LS, little is known about the genetic alterations and mechanism of carcinogenesis behind this form of CRC.

CRC and emergency presentation

As discussed previously 15-30% of CRCs present themselves as emergency cases, most often due to obstruction (78%), perforation (10%) or bleeding (4%) [40, 41]. The most common sites for tumor obstruction are the left colon and the sigmoid [173, 174] which is in line with the smaller luminal diameter and more solid fecal content in the left side of colon compared to the right. The risk for obstruction seems to be highest at the splenic flexure [173, 174]. The most frequent sites for perforation are reported to be the sigmoid and caecum [175].

Patients undergoing acute surgery are older than the elective ones (mean age 68.6 years compared to 66.3 years). Both young patients (<40 years) and old patients (>80 years) with CRC more often present as emergencies, probably because both groups are at risk of having their symptoms ignored. Some reports have shown a female predominance, but the role of estrogen in this setting is yet to be defined [41, 176].

Many studies report poorer outcomes for patients who undergo emergency surgery, both during their initial hospital stay and their long-term survival [40, 41, 176]. Acute and severe disturbances of body physiology may explain the differences in short-term perioperative survival. Emergency CRCs have been associated with a higher risk of metastatic disease, possibly because of occult liver metastases already at the time of surgery, although not necessarily showing a higher rate of local recurrence [173, 176]. In one study, the five year overall survival for emergency patients was 39.2% compared to 64.7% for elective cases [41] and a median survival time of 59 months compared to 82 months has been reported [177]. Advanced tumor pathology and tumors with unfavorable histologic features may provide the basis for the differences in outcome. Emergency patients tend to have more advanced cancers (AJCC stage III and IV) and more T3 and T4 tumors as well as a higher rate of N1 and N2 cases, compared to elective patients. According to some studies, on a stage-for-stage analysis, the survival rates remain worse for emergency cases, even after substratification for factors such as lymph node status and presence of extramural lymphovascular invasion [41, 177]. Positive resections margins are also more frequent among cases presenting as surgical emergencies [177].

Several studies have found no differences in the morphological profile of emergency and elective CRCs [173, 178-180]. Extramural venous invasion, however, has been reported as being more common in emergency cases [177]. In one study perforated tumors were found to present more often with distant metastases, although they were more seldom poorly differentiated and had less lymph node involvement than non-perforated cases [181]. The

findings are contradictory and difficult to interpret but might represent differences between emergency and elective cases in the molecular features that lie behind hematogenous and lymphatic spread.

Summary

As presented above, the histopathological profile of CRC seems to show considerable variation in relation to sex, age, tumor location, family history and mode of presentation, although the biological background for this is still largely unclear. These findings could however speak for different mechanisms of tumor development in men and women, young and old patients, proximal and distal colon, sporadic and familial cases and elective and emergency CRCs. Since many of the genes involved in CRC carcinogenesis are morphogenes, i. e. genes that have major influence on cell and tissue morphology, differences in tumor phenotype could reflect differences in the underlying genetic contribution.

6. AIMS OF THE THESIS

The overall premise of this work is the notion that tumor morphology could reflect the genetic contribution or underlying tumorigenic mechanisms. Although the underlying mechanism itself might not be elucidated, identifying histopathological differences between different groups of tumors will support the idea of different etiological backgrounds in these groups.

The specific aim of each paper was:

Paper I

To determine whether 11 newly identified genetic susceptibility loci were associated with tumor morphology to confirm them as distinct and etiologically different risk factors in colorectal carcinogenesis.

Paper II

To provide a detailed and systematic histopathological characterization of CRC in a large population-based cohort, by comparing the morphology of tumors in men and women, in different age groups, in different anatomical locations, and in sporadic and familial cases, in order to isolate the effects of these four factors.

Paper III

To compare the clinicopathological profile of emergency and elective cases of CRC in relation to sex, age groups, location, and family history of CRC.

Paper IV

To use the family history of cancer in patients with CRC together with genotyping and tumor morphology in order to find support for and define new CRC syndromes.

7. MATERIALS AND METHODS

Materials

Patients

All patients in studies I-IV where recruited within the Swedish Low-Risk Colorectal Cancer Study which was design to identify both new high-risk genes in families with strong inheritance for CRC as well as putative low-risk alleles in a population based material of CRC. This study, initiated by Professor Annika Lindblom, Karolinska Institutet, recruited patients consecutively operated for CRC during 2004 to 2006 from 14 different surgical clinics in Mid-Sweden (the regions of Stockholm and Uppsala). Of 4585 patients operated during this time period, 2175 (47.7%) were included in the study. The corresponding figures for Stockholm County were 2573 and 1205 (46.8%). Patients who were too old or too ill to be invited were excluded; otherwise all patients were eligible. Of the 2410 patients that were not included, 639 died before they could be asked to participate or before blood could be drawn. The rest declined to participate, withdrew their consent or were excluded for various reasons.

For the comparison of emergency and elective cases (paper III) only patients from Stockholm County were selected. The reason for this was that the medical records from which we gathered information about the type of operation were easily available to us. For further details see the Materials and Methods section in each paper I-IV.

Histological specimens

For all patients in studies I-IV an attempt was made to obtain the original H&E-stained slides of tumor(s) from the pathology department involved, as well as the original pathology report. When slides could not be found in archives new sections were prepared from paraffin blocks if possible. In 0.4% of cases only biopsy material was available and in 2.0% the specimen consisted of a polypectomy or local resection. In the rest of the cases assessment was made on the surgical specimen.

Although all patients examined in studies I-IV originated from the same cohort the exact number of reviewed cases stated in each paper varies slightly. In paper I, the number of included cases (n=1572) refers to the number of patients were a surgical specimen could be re-reviewed, where blood could be drawn, where the family history was known and where cases of FAP and LS were excluded. In paper II, the number of included cases (n=1613) refers to all patients with a surgical specimen available. In the analysis of morphology in relation to family history those with unknown family history and cases of FAP or LS were of course omitted. In study IV patients were consecutively included with an arbitrary cut-off at 1720 patients (rendering 1612 available specimens), mainly to allow for the histological assessment to be finished in time.

Methods

Histopathological assessment

All tumors included in study I-IV were re-reviewed in the same way according to a standardized protocol. Tumor location and information about multiple synchronous tumors was gathered from the original pathology report as well as the Regional Oncologic Center registry. Information on whether the patient had received pre-operative chemo- and/or radiotherapy (for rectal cancers) was obtained from the clinical history on the pathology referral sheet and from lists provided by the Regional Oncologic Centers.

The micromorphological parameters assessed were tumor grade, stage, medullary features, mucin production, mucin type, Crohn-like peritumoral lymphocytic reaction, tumor infiltrating lymphocytes (TILs), desmoplasia, tumor necrosis, vascular invasion, perineural growth, co-existing polyps, budding and type of tumor margin. For the exact definition of these features and how they were assessed see the Materials & Methods section in paper I or II.

Genotyping

All cases in study I were genotyped for one SNP from each CRC risk locus: rs16892766 on 8q23.3, rs6983267 on 8q24.21, rs719725 on 9p24, rs10795668 on 10p14, rs3802842 on 11q23.1, rs4444235 on 14q22.2, rs4779584 on 15q13.3, rs9929218 on 16q22.1, rs4939827 on 18q21.1, rs10411210 on 19q13.1, and rs961253 on 20p12.3. In study IV the cases were genotyped for only rs6983267. Six of the SNPs (rs719725, rs4444235, rs4779584, rs9929218, rs10411210, and rs961253) were typed using TaqMan SNP genotyping assay (Applied Biosystems, Foster City, CA). Genotyping of the remaining five SNPs (rs6983267, rs16892766, rs10795668, rs4939827, and rs3802842) were performed using a technology developed by Nanogen, at deCode Genetics, Reykjavik, Iceland.

Statistical analyses

In study I the cross tabulation between SNP data and morphology was done and Pearson $\chi 2$ test was used for calculating the p-value. The significant results from these genotype–phenotype analyses were studied further by using the DeFinetti program (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl). Deviations of genotype frequencies in cases and controls from those expected under Hardy-Weinberg equilibrium were calculated by $\chi 2$ tests (one degree of freedom). Odds ratios, 95% confidence intervals, and the corresponding p values were calculated using the same program. Results are presented without correction for multiple testing to avoid the loss of valuable information due to the limited number of patients.

In studies II and III determination of the association between clinicopathological features and sex, age group, location, family history (and type of operation in study III) was performed by univariate and multiple binary and multinomial logistic regression analysis for categorical outcomes and linear regression analysis for continuous outcomes. Results

are presented as odds ratios (ORs) from the logistic regression and as regression coefficients (b) from the linear regression. In addition, factor analysis (extracting factors using principal components analysis) with variance maximizing (varimax) rotation was performed to form a concise description for all the variables included in the study.

In study IV Mann-Whitney U-test, Students T-test and Speaerman´s rank-order analysis were used. Correlation between syndromes and morphology was investigated using cross tabulation-analysis and Pearson $\chi 2$ test.

The significance level for all statistical tests was set at 0.05 but also non-significant p-values were recorded.

8. RESULTS

Paper I

Of the 11 tested loci (SNPs) six showed statistically significant correlations to morphological parameters and a total of 10 genotype-phenotype associations were significant. After the DeFinetti analysis (to obtain the ORs and confidence intervals) five SNPs remained significantly associated with morphological parameters (see Table 1 in paper I).

Heterozygous carriers of the T allele of rs6983267 (8q24.21) had decreased Crohn-like peritumoral lymphocytic reaction (p=0.021). For rs10795668 (10p14), the heterozygote genotype was associated with poor differentiation (p=0.015). Homozygosity for the C allele of rs4444235 (14q22.2) was related to decreased Crohn-like peritumoral reaction (p=0.024). The T allele of rs10411210 (19q13.11) was negatively associated with desmoplastic response (p=0.004 for homozygotes). For rs961253 (20p12.3), the variant A allele was associated with mucin producing tumors (p=0.010 and 0.009 for homozygotes and heterozygotes respectively). Homozygous carriers of the A allele more frequently had tumors with circumscribed margins (less often infiltrative, p=0.034) but for heterozygous carriers an opposite effect was suggested.

Paper II

The univariate comparison between men and women (Table 1 in paper II) showed that female patients significantly more often had tumors with TILs >30/10 high-power fields (HPF) and tumors of medullary type. Women also showed a lower frequency of tumors with an infiltrative margin. In the multivariate analysis (Table 2 in paper II) a significant difference remained only in TILs (p=0.002).

The univariate comparison between the three age groups (Table 3 in paper II) showed that patients aged <60 years had a significantly lower frequency of multiple tumors, mucin production (0–50% mucin), and TILs >30/10 HPF compared to the reference group (>75 years). They, however, showed a higher frequency of AJCC stage III tumors, N1 and N2/N3 tumors, vascular and perineural invasion, and infiltrative tumor margin. In the multivariate analysis (Tables 2 and 4 in paper I) significant differences remained for multiple tumors, AJCC stage III, N2/N3, perineural invasion and infiltrative tumor margin, which had the highest level of significance (p<0.0001). In addition, AJCC stage II and IV tumors and T4 tumors were significantly more common in the youngest age group.

In the univariate comparison (Table 5 in paper II) most of the histological features studied showed a significant difference between the left colon and rectum compared to the right colon (reference group). The most significant differences between the left and right colon

were seen in mean tumor diameter, T3 tumors, proportion of poorly differentiated tumors, mucin production, mucinous type (>50% mucin), TILs and medullary type. All of the differences from the univariate analyses, except for the higher frequency of N2/N3 tumors in the left colon, remained significant in the multivariate analyses, where the highest level of significance ($p<0.0001$) was seen for tumor diameter, proportion of poorly differentiated tumors, Crohn-like reaction, TILs, medullary type, T3 tumors, and mucinous type. In the univariate comparison between rectum and right colon, most of the features listed in Table 5 in paper II showed highly significant (<0.0001) differences and all of these remained significant in the multivariate comparison.

The only difference between the sporadic and the familial group was seen in vascular invasion, which was more common among the familial cases ($p=0.012$ in the multivariate analysis, Table 2 in paper II).

All the dependent and independent variables could be grouped into six different factors (components) according to Table 7 in paper II.

Paper III

The univariate comparison between elective and emergency cases (Table 1 in paper III) showed that the emergency cases had significantly more often multiple tumors, vascular invasion, perineural invasion and infiltrative tumor margin. There was no difference in mucin production, but the tumors in the emergency group more often showed a signet-ring cell component. Also, the emergency patients had more AJCC stage II-IV tumors than stage I tumors, compared to the elective group. They also had higher T- and N-stage, but more seldom TILs>30/10 HPF. In the multivariate comparison (Table 2a and b in paper III) together with sex, age group, location and family history, type of surgery remained a significant factor for multiple tumors, vascular invasion, perineural invasion, tumor margin, mucin type (signet ring cell component vs. only extracellular), AJCC-stage, T- and N-stage and TILs. The highest level of significance ($p<0.0001$) was seen for multiple tumors, perineural invasion, infiltrative tumor margin, AJCC stage III vs. I and N1 and N2/3 vs. N0.

In both univariate and multivariate analysis of the effect of sex, age group, location and family history on the type of surgery, the only significant result was seen for location where there was a much lower risk of having to undergo emergency surgery for a rectal cancer compared to a right sided colon cancer ($p<0.0001$ in the multivariate analysis).

All the dependent and independent variables could be grouped into seven different factors (components) according to Table 3 in paper III.

Paper IV

When comparing the number of cancers between the families of the sporadic and familial CRC cases (Table 1 in paper IV), there were significantly more cancers of all types in the

family history of the familial cases of CRC ($p<0.001$) and also significant more bladder cancer ($p<0.001$), prostate cancer ($p<0.01$), melanoma ($p=0.01$), gastric cancer ($p<0.01$).

Testing the SNP rs6983267, already known to be associated with both CRC and prostate cancer, confirmed this SNP to be more common in families with both colorectal and prostate cancer ($p=0.017$).

An analysis of the CRC morphology in the index case in relation to the different suggested syndromes gave some support for different morphological profiles in four of the five tested syndromes (Table 2 in paper IV). The CRCs in the cancer families (families with at least two CRCs and three or more other types of cancer, in first- or second-degree relatives or cousins) displayed more often vascular invasion. The tumors in the CRC and prostate cancer families were associated with budding and these patients also more frequently had lymph node metastases. The cases from CRC and gastric cancer families more often had tumors with Crohn-like peritumoral lymphocytic reaction. Finally, CRC cases in families with CRC and melanoma showed association to poor differentiation.

9. DISCUSSION

Paper I

In this study we have demonstrated a unique pattern of morphological parameters associated with five recently published low-risk gene variants of CRC located on chromosomes 8, 10, 14, 19 and 20. The susceptibility region on 8q24.21 (rs6983267) has previously been associated with an elevated risk of adenoma development as well increased risk of prostate cancer [111, 115]. Also, this SNP has been related to family history, MMR status, tumor site and tumor stage [182]. In our study, heterozygosity for the variant allele (T) in this locus was demonstrated to be negatively associated with Crohn-like peritumoral lymphocytic infiltration, a host immune response that has been linked to improved patient survival in some studies [125, 126]. Therefore, a five-year follow up of our patients would be interesting and could perhaps reveal if variations in this SNP are related to outcome.

For rs719725 (9p24) the results for desmoplastic reaction, budding, and necrosis were inconsistent in homo- and heterozygous carriers; heterozygotes for the variant allele (C) seemingly having an increased risk and homozygotes a decreased risk. Although showing significant p-values, we therefore regarded these results as false positive and unlikely to be associated with any of the studied phenotypes. Homozygosity for variant allele (T) of SNP rs10411210 on 19q13.11 was negatively associated with desmoplastic reaction. This feature is generally considered favorable [130], although there are conflicting reports regarding the role of fibrotic stromal response in cancer development and whether it favors the host or the tumor [131]. Also in this case, a five-year follow up of our material could be of interest. The region on 20p12.3 harbors a risk allele (A) associated with mucin-producing tumors. Mucinous tumors have been showed to confer a poorer 5-year survival compared to non-mucinous CRCs [145]. Many mucinous carcinomas are however MSI-H positive and thereby low-grade and carrying a better prognosis [148]. In addition, homozygous carriers of the A allele showed an association to tumors with circumscribed margin. However, for heterozygous carriers the results suggested an opposite effect making interpretation of this finding difficult.

Heterozygosity for the variant allele (A) at the 10p14 locus, reported to have a protective effect against CRC, was associated with poorly differentiated tumors but with no other MSI-like morphology. Similar to the locus on 8q24.21, the 14q22.2 locus harbors an allele negatively correlated to Crohn-like peritumoral lymphocytic reaction. However, for the variant on 8q24.21, it is the allele providing a protective effect (T) that is associated with this tumor phenotype, while for the variant on 14.q22.2, it is the risk allele (C).

The studied SNPs have pointed out regions associated with morphological features, but it is difficult to interpret some of these correlations in their biological context as the exact pathogenic variation is still not known for all risk loci. However, the 8q24.21 locus has been demonstrated to affect the last nucleotide of a binding site for TCF4, thereby up-regulating the oncogene *MYC*, which might explain some of the increased risk of CRC for

carriers of the risk allele (G) [116]. The closest gene to 20p12.3 is *BMP2*, and similarly, *BMP4* maps close to the 14q22.2 locus [183]. Both these genes belong to the TGFβ-family, which is a morphogenic factor involved in CRC carcinogenesis as discussed in Chapter 2. For the locus 10p14, there is no coding transcript or predicted gene within 0.4 Mb of sequence from the SNP [122]. The 19q13.1 locus maps to a 96-kb block of linkage disequilibrium that contains the gene *RHPN2*, suggested to be involved in in the biology of invasiveness of CRC [184].

In a study such as this where many tests have been performed the problem of multiple comparisons must of course be addressed, although the usual Bonferroni correction might be too strict. Since it is not clear what the appropriate correction needs to be and since this is the first study of detailed morphology associated to CRC low-risk alleles, we thought it was important to show all possible results for future comparisons.

In the study of cancer as a complex disease, it is expected that numerous genes and pathways will act together and that this will influence risk effects. The effect of each individual genetic variant above has been demonstrated to be extremely small with relative risks only just above 1. Hence, understanding the genetic effects on function as seen by clinical parameters such as tumor phenotype is important. That cancer-causing genes do influence morphology has been shown from the study of high-risk genes [185]. With regard to this, it would be interesting to add immunohistochemical profiling to our study and relate the outcome of this to the various SNPs studied here. This immunohistochemical panel could for example include expression of proteins coded for by genes located close the SNPs described above (MYC, BMP2 and 4, and RHPN2), but also other proteins important in CRC tumorigenesis such as KRAS, BRAF, SMAD2, 4 and 7, β-catenin, p53, TGFβ-receptors and MMR-proteins. Molecules involved in cell adhesion, invasiveness and metastatic potential such as E-cadherin, CEA, MMPs, VEGF and PD-ECGF could also be included in the marker panel, together with cytokeratins, CDX2 and mucin stains.

In summary, the knowledge of genes or genetic variants involved in cancer development has future clinical potential in prevention, diagnosis, and prognosis and even for decisions regarding therapeutic strategies. However, our results are preliminary, and more studies are required to confirm these findings. In particular, a long-term follow-up would be important to evaluate the survival implications related to the investigated risk alleles.

Paper II

Sex

Tumors with TILs>30/10 HPF, medullary features and circumscribed margin were more common in women than in men, although in the multivariate analysis only the difference in TILs remained significant (OR 1.482, p=0.002). A high number of TILs, medullary features and circumscribed tumor margin are all features associated with MSI-H tumors. The results support previous studies that have shown cancers with MSI-H phenotype to be more common in women than in men [80, 81]. Differences in hormonal status could be a possible explanation. There is clinical evidence that estrogen protects against the

development of CRC, but its exact role in the carcinogenesis is not well understood. Exogenous estrogen has been associated with the prevention of hypermethylation-associated loss of estrogen receptors, which can lead to unregulated growth of the colonic mucosa [186]. At least three different estrogen receptors, ERβ1 (estrogen receptor β1), ERβ2 and ERβ5 have been detected in normal and malignant colorectal epithelium. Studies have shown that ERβ1 and ERβ2 expression is lost in many CRCs. High ERβ1 expression is associated with low-grade carcinomas, lower T-stage, mucinous phenotype and MSI. High ERβ2 expression is found in carcinomas with right-sided location and those with lymph node metastases. Loss of ERβ1 is thereby associated with more aggressive CRCs, whereas the opposite is true for ERβ2. It has been proposed that ERβ1 activation predisposes to MSI and that such activation is somehow suppressed by estrogen before the menopause. Estrogen withdrawal will lead to a rebound increase in ERβ1 expression and thereby a higher risk of MSI-H carcinomas in older women [187]. This is in line with older women having more MSI-H cancers compared to younger women, in contrast to men, where the frequency of MSI cancers decreases with age [160, 188].

Age

When comparing CRCs in different age groups we chose cut-off points at 60 and 75 years in order to get three groups of comparable size. Multiple synchronous tumors were clearly much less common (OR 0.204, $p<0.003$ in the multivariate analysis) in the youngest group (<60 years) compared to the reference group (>75 years). The results suggest that age is a crucial factor for this feature. This may be due to young patients having a better anti-tumorigenic immune response, which prevents them from developing multiple cancers. Also, they may not yet have accumulated as many mutations in their colonic mucosa as older patients. Alternatively, the tumors of the young patients may be more fast-growing so that they will cause symptoms and be diagnosed before additional tumors have developed. Interestingly, patients aged less than 60 years showed more locally advanced tumors with more vascular and perineural invasion and infiltrative tumor margin. They also showed higher ORs for AJCC stage II–III, T4 and N2/N3 tumors, than the reference group. The results indicate that younger patients have a more aggressive disease, which is in line with some previous reports [163, 165], but in contrast to others [164]. When looking at the univariate analysis, the tumors of the young patients displayed less mucin production, less Crohn-like lymphoid reaction, more seldom medullary features, and had a lower frequency of TILs. These features constitute the opposite of the MSI phenotype seen in older patients [80]. The finding of less mucin production is in contrast to reports showing mucinous tumors to be more frequent in young patients [164]. None of these features, however, remained significant in the multivariate analysis. All in all, the patient's age seems to be correlated to tumor aggressiveness, rather than to morphology. The tumors of the young patients were more systemically advanced by the time of operation, thus indicating faster growth.

Location

Multiple tumors were much less common in the rectum than in the right colon (OR 0.308, $p<0.0001$ in the multivariate analysis). This is probably for anatomical reasons: the short length of the rectum and the narrow lumen result in symptoms and early discovery before

any possible additional tumor could develop. The same anatomical factors probably explain why the tumors in the left colon and rectum were smaller than the tumors in the right colon. In addition to the larger lumen of the right colon, its bowel contents are also looser, which makes tumors in this location escape early detection by not causing symptoms such as obstipation. The tumors in the rectum, and to a certain extent in the left colon, tended to be of lower AJCC- and T-stage than those in the right colon. This characteristic might also be explained by the fact that these tumors are detected earlier.

Mucinous tumors were more common in the right colon compared to both the left colon and the rectum. Because mucin production is part of the morphological spectrum of MSI-H tumors, which are more common on the right side, this is not surprising. The same was true for tumors with a high number of TILs and medullary features, which are also characteristics of MSI cancers. The frequency of signet-ring cell morphology parallels that of mucin production as a whole, with tumors showing this feature being significantly more common in the right colon. As discussed in Chapter 4, signet-ring cell carcinomas are known to present themselves at a higher stage, confer a poorer prognosis and show a different pattern of genetic changes compared to conventional adenocarcinomas. Rectal tumors showed more perineural invasion, and an infiltrative tumor margin was more frequent in both rectal and left-sided cancers, compared with findings in right-sided cancers. Again, anatomical factors may lie behind this difference, as the rectum, which mainly consists of an outer longitudinal muscle without haustrae and with its own mesentery, is innervated by a surrounding plexus of sympathetic and parasympathetic fibers. This, in turn, results in a high concentration of nerves close to the wall of the rectum. The limited space for luminal expansion in the rectum and left colon – because of the smaller diameter – may also force tumors in these locations to grow outward, hence causing a more infiltrative pattern. For most morphological parameters the differences seem to be greatest between right-sided colon cancers and rectal cancers. In addition, most features show a gradient from right colon to left colon to rectum, as indicated by the ORs.

Most of the morphological parameters studied seem to be related to tumor location rather than to age-group according to the multivariate analysis. This is interesting since there are several embryological, environmental and genetic differences between different parts of the large bowel. Proximal colon originates embryologically from the midgut, while distal colon and rectum originate from the hindgut. Histologically the epithelial cells of proximal colon contain dense mucous apical vesicles, while the proportion of goblet cells is highest in distal colon. Rectum on the other hand shows a high concentration of endocrine cells. The bacterial fermentation products in proximal colon are rich in short-chain fatty acids and ethanol, while products of protein fermentation dominate distally. Proximal cancers are more related the MSI pathway and the CpG methylator phenotype, while in distal cancers the CIN pathway with mutations in *KRAS*, *APC*, *TP53* and *DCC/SMAD4* is predominant. Rectal cancers are rarely MSI-H positive, whereas the incidence of CIN is high. However, compared with colon cancers, rectal cancers show a significantly higher number of mutations. Higher expression of nuclear β-catenin, p53 and COX2 is also seen in rectal cancers compared to colon cancers [189].

Sporadic vs. familial

There were remarkably few differences in the morphology between sporadic and familial CRCs. Familial CRCs, however, showed a higher frequency of vascular invasion (OR 1.438, p=0.012 in the multivariate analysis). 27.4% of the familial cases displayed this feature, compared to 21.1% of sporadic cases. Considering the retrospective nature and the size of the study, as well as cost-, time-, and labor-related aspects because of additional immunohistochemistry, we chose not to differentiate between venous and lymphatic invasion. Given the problem with low reproducibility, high interobserver variability and high false negative rates as discussed in Chapter 4, our rate of vascular invasion, which is in the lower range of previously reported frequencies of 10 to 89.5% [137] might represent an underdiagnosis of this feature.

The finding of a higher frequency of vascular invasion in familial tumors however raises the question of whether tumors in the familial group have different biological properties, such as specific tumor antigens or adhesion molecules that influence the ability to invade vessel walls. Protein markers such as apoptosis protease activating factor-1 (APAF-1), mammalian sterile 20-like kinase (MST1), urokinase plasminogen activator receptor (uPAR), Raf-1 kinase inhibitor protein (RKIP) and VEGF have been associated with vascular invasion [190]. The urokinase plasminogen activator (uPA)/uPAR system is associated with the degradation and regeneration of the basement membrane and extracellular matrix and uPAR itself is involved in cell movement and adhesion. *RKIP* has recently been characterized as a metastasis suppressor gene and loss of it has been associated with an increased frequency of distant metastases in CRC [190]. All in all, our finding may speak for a difference between sporadic and familial CRCs in the expression of proteins facilitating vascular invasion, but extensive immunohistochemical comparison, including some of the above mentioned markers, of the two groups is required. One could expect that differences in vascular invasion between the two groups would be reflected in N stage. However, no such difference was evident. A higher frequency of vascular invasion should feasibly lead to more distant metastases, but M stage was not possible to assess in our material. A follow up of our patients after 5–10 years could perhaps reveal a correlation between vascular invasion and survival time, as has been shown in previous reports [190, 191].

Factor analysis

We found that AJCC- and N-stage were in the same component (factor 1) together with vascular invasion, perineural invasion, budding, and tumor margin. This is not surprising because these are all features related to the extent of tumor spread and tumor aggressiveness. T-stage had a meaningful loading on two components and was therefore ignored in the interpretation. Mucin and mucin production were grouped in the same component (factor 2). Crohn-like peritumoral lymphocytic infiltrate is part of the MSI spectrum, but in our analysis it was not grouped in the same component (factor 3) as the other MSI variables grade of differentiation (negative correlation to well/moderate), TILs, and medullary type. This finding supports the fact that peritumoral lymphocytic infiltration is a different entity from TILs and that it may have a different biological implication. Desmoplastic reaction and Crohn-like peritumoral lymphocytic infiltration

were grouped together with tumor diameter (factor 4). The fifth component (factor 5) consisted of age group and multiple tumors. This is in keeping with the multivariate analysis which showed that patients younger than 60 years had significantly fewer multiple tumors than the reference group. In addition, our factor analysis showed a sixth component (factor 6), consisting of sex and family history. (Please note the error regarding this in the Factor analysis section under Discussion in paper II). Location had a meaningful loading on both factors 4 and 5 and was therefore ignored; however, this loading was not so high, -0.41 and -0.44, respectively.

In summary, we have in this large and systematic study shown that tumor location is the factor having most influence on morphology. The results are in line with tumors in different locations having different genetic and embryological backgrounds as well as developing in different physiological settings. Age is the most important determinant for the presence of multiple tumors and an important factor for the aggressiveness of the disease. The results could speak for different mechanisms of tumor development in young and old patients. Few morphological features are related to sex and almost none to family history. The observed morphological differences in our material could perhaps be supported by immunohistochemical markers as outlined in the discussion about paper I, in a subset of the patients. The prognostic significance of our findings must, however, await a 5 to 10 year follow-up.

Paper III

According to our study emergency cases of CRC more often show multiple tumors (OR 3.154, $p<0.0001$ in the multivariate analysis). This seems reasonable since multiple tumors should increase the risk for obstruction. Emergency tumors tend to be of higher AJCC-stage (II-IV), T-stage (T4) and N-stage (N1-2/3), which is in line with previous reports [41, 177] and not surprising since T-stage and AJCC-stage reflects how locally advanced the tumor is. It seems reasonable that locally advanced tumors by growing through the bowel wall could be more prone to perforation. A locally advanced cancer would also be more likely to display vascular and perineural invasion, which is in fact shown in our material (OR 2.086, $p=0.001$ and OR 2.500, $p<0.0001$ respectively in the multivariate comparison). Vascular invasion in turn, would increase the probability of lymph node metastases as indicated by the N-stage.

Interestingly, there was no difference in tumor diameter between the emergency and elective group. Nor was there any difference in the frequency of mucinous tumors or tumors showing necrosis. One would expect large, mucinous or necrotic tumors to more easily cause obstruction or perforation resulting in emergency surgery. However, the perforations associated with colonic cancer are mainly due to a direct mechanism of local destruction at the site of the cancer which does not necessarily mean that the tumor itself has to reach a certain size to achieve that. Also, in about one third of the perforated cases the perforation is located proximal to the cancer [192]. In this situation, which is well-known by colorectal surgeons, a diastatic widening occurs in the cecum eventually creating a perforation. This is often the case in left-sided (sigmoidal) tumors. Due to the consistency of the stools in this region these cancers are prone to cause an obstruction which in turn

will widen the proximal part. The law of La Place states that the site of largest diameter requires the least pressure to distend. Hence, cecum is the most vulnerable part and will perforate at a certain diameter, described as 13 cm in the literature [193], due to a distal cancer in the left colon. Rectal cancers seldom present as emergencies (5.9%) compared to colon cancers (21.7%) [41], which is in line with rectal tumors causing early symptoms and being detected before they become advanced enough to cause obstruction.

The emergency group showed more frequently mucinous tumors with signet-ring cells (OR 3.136, p=0.001 in the multivariate analysis). This type of mucin production with mucus pools filled with cells displaying a large cytoplasmatic mucin vacuole could make the tumor less cohesive and more soft and thereby more prone to perforation. We found tumors with TILs>30/HPF to be less frequent in the emergency cases compared to the elective ones. As discussed previously, TILs is a distinct feature of MSI-H tumors. About 30% of right-sided CRCs are shown to be of MSI-H type and the majority of MSI-H tumors are located on the right side [194]. The most common site of obstruction has been reported to be the sigmoid [174] which might explain the underrepresentation of tumors with high number of TILs among the emergency cases. Irrespective of the MSI status, the invasion of lymphocytes could reflect antitumor immunity [128] and in emergency cases leading to perforation this cellular reaction might not be developed. Three MSI-associated features, multiple tumors, signet-ring cell carcinomas and Crohn-like lymphocytic reaction, were more common among the emergency cases while a high number of TILs and circumscribed tumor margin was more frequent among the elective cases. No difference was seen in poor differentiation, mucin production or medullary tumors which are also included in the MSI spectrum. Thus, in sum MSI-H features of CRC did not appear to predominate in either the emergency or elective group.

Vascular invasion, as mentioned above, was more common among the emergency cases in our material. This is in line one previous report which showed extramural venous invasion to be more frequent in this group [177]. It seems likely that emergency tumors being more locally advanced will show a higher frequency of both vascular and perineural invasion. This is probably also reflected in those reports showing a worse prognosis for emergency cases [40, 41, 176]. The emergency cases also displayed a higher frequency of tumors with infiltrative margins (OR 2.452, p<0.0001 in the multivariate comparison), which is in accordance with the fact that locally aggressive tumors could cause perforation. When looking at the effect of sex, age group, location and family history on type of surgical presentation, only location turned out to be a significant factor with a clearly lower risk of having to undergo emergency surgery for a rectal cancer compared to a right sided cancer. This finding is not surprising and is in line with the clinical appearance of rectal cancer and its surgical management.

In the factor analysis AJCC- and N-stage were in the same component (factor 1) together with vascular invasion, perineural invasion and tumor margin. As discussed in paper II these are all features related to extent of tumor spread and tumor aggressiveness. Mucin production and mucin type were grouped into the same component (factor 2). Grade of differentiation (negative correlation to well/moderate), number of TILs and medullary

type are all features related to the MSI-H phenotype of CRC (factor 3). Crohn-like peritumoral lymphocytic infiltrate, which is also an MSI-feature, was however not included in this factor. Tumor diameter and desmoplasia were grouped together (factor 4). Factor 5 included location and peritumoral lymphocytic infiltration. This is in accordance with our previous observation in paper II that the frequency of peritumoral lymphocytic reaction is higher in right-sided CRCs. Family history and multiple tumors were grouped together (factor 6) and budding separately (factor 7).

All in all, emergency CRCs in general show a more aggressive histopathological profile and more advanced stage, than elective CRCs. Since the distribution of emergency and elective cases was essentially similar between right and left colon the observed differences cannot primarily be attributed to differences in macroenvironment or location between the two groups. This raises the question whether CRCs presenting as emergencies may have a different etiological or genetic background. The well-known fact that emergency colorectal surgery is associated with a worse outcome, including higher morbidity and relapse, has traditionally been characterized mainly as a technical and surgical problem. Discussion about surgery in an emergency situation under conditions less optimal and sometimes by a surgeon who is not necessarily specialized in colorectal surgery, has dominated the debate. This has led to a more frequent use of adjuvant chemotherapy in the postoperative care. Our study suggests that the complexity of the issue probably involves a more aggressive biology of the tumor itself. If future studies could classify the genetic background of these tumors a more precise and adequate oncologic treatment might be offered. Using SNPs to pinpoint chromosomal loci associated with an emergency phenotype and looking at genes located in or close to these loci could provide an insight into which pathways are involved in emergency contra elective cases. As suggested in paper I and II, immunohistochemical studies especially focused on markers for invasion, loss of cell adhesion, metastasis and proliferation rate (Ki67), could also help to further explore the eventual differences between the two groups. Furthermore, in our study we have not separated obstructive and perforated lesions. It seems reasonable that the two types of emergency tumors might show differences in morphology and/or immunohistochemical profile which could be addressed in a future study.

Paper IV

Known cancer syndromes often involve an increased risk for a whole spectrum of tumors, such as CRC, endometrial, gastric, renal pelvis and ileal tumors in LS and breast cancer, leukemia, sarcoma, and brain tumors in Li-Fraumeni syndrome. Also for the BRCA genes, the VHL, the APC and in fact almost all known cancer genes, a typical spectrum of different cancers is associated with each gene involved in the syndrome.

When CRC cluster in families where none of the known syndromes are prevalent also other tumors are frequently seen. To find out if this was significant, we used a cohort of consecutive CRC cases and their family history of cancer among close relatives for the

study. After comparison of the family history it was clear that several tumors were more prevalent in the families with more than one CRC case. It is difficult to determine whether there was only one cancer family syndrome, with a differently increased risk for most cancers, or several – including a number of different tumor spectra. We tested each tumor type separately and found positive values for urine bladder, prostate and gastric cancer and melanoma. It is possible that only one cancer syndrome is responsible for the results and that some tumors show a positive correlation because they are common enough to give power on their own. However, urine bladder carcinoma is quite rare and still gives a positive correlation – while breast cancer, which is very common, does not seem to be more frequent in the familial group. Thus, there is likely at least some kind of specificity for one or several CRC syndromes but without the knowledge of underlying genetic contribution it is impossible to say which tumors are associated with which syndrome.

One limitation of our study is that many of the diagnoses among family members were not verified from medical records. However, all abdominal malignancies with unclear diagnosis were verified in order to confirm or exclude CRC. Other diagnoses were coded as reported from the index patient if stated in detail and claiming good knowledge. Weak remembrance or uncertainty did not result in coding of a cancer diagnosis. Some malignancies were considered more uncertain than others. So, i.e. gynecological malignancies and hematological malignancies are often stated as such and only rarely specified in detail why we chose to use these terms for all cases reported regardless of how specific the diagnoses were expressed.

The MSI status was not known to us, why we could not predict LS in a better way than by using the Amsterdam II criteria. Only about 1.2% of the patients in Sweden should have LS judging from a previous study [93]. Such a small proportion is not likely to have influenced our results. Considering the results and the suggested syndromic tumors, only gastric cancer is associated with LS. Urinary bladder cancer has not been considered associated with LS, where cancer in the renal pelvis is seen, although rarely at all in Swedish families. Melanoma has not been reported to be overrepresented in LS. Quite recently a Norwegian study reported prostate cancer to be more common in LS-gene carriers than among the general population [195]. Gastric cancer and gynecological cancer constitute typical tumors of the LS. However, in Sweden gastric cancer is rarely seen in LS families and endometrial cancer is often associated with CRC in non-LS families, why none is typical for LS in our experience (Annika Lindblom. Unpublished).

An effort was made to find more evidence in support of the new syndromes suggested. Two different approaches, molecular genetic studies and studies of tumor morphology were used. Since the family history studies included all diagnoses on both the maternal and paternal side, both monogenic syndromes and complex inheritance – or both – could explain our findings. The monogenic syndromes will be tested for in future linkage analysis in families suggested to have monogenic disease as outlined in this study. However, we could immediately test the hypothesis of complex disease by choosing the SNP rs6983267 published to be associated with an increased risk of both prostate cancer and CRC, as discussed previously. We found support for this SNP to be associated with

an increased risk in families with both CRC and prostate cancer (p=0.017) which demonstrates a molecular evidence for at least one of the syndromes suggested.

CRC predisposing genes are typically morphogenes and thus CRC tumors will demonstrate different morphology depending on the underlying genetic contribution [185]. Tumor morphology and location of the tumors in the index cases were used for testing the hypothesis that tumors in the different syndromes might show different and typical phenotypic characteristics to support different underlying genetic etiology. We found statistically significant associations for four of five tested hypothetical syndromes; cancer families, CRC-prostate cancer families, CRC-gastric cancer families and CRC-melanoma families. The findings included only one of 15 tested tumor characteristics each, why this is not strong evidence for any of the syndromes. However, it still gives some support for a different genetic underlying cause of those syndromes.

In conclusion, we used the family history of cancer in relatives of consecutive CRC patients to define putative new CRC syndromes. Some supportive evidence was also found by genetic association and morphological analysis. The rationale for this report was to define new syndromes that could be used for future studies of finding new predisposing genes. Further studies aiming to find the underlying genetic contribution must be undertaken to test these hypothetical syndromes, including replication of the syndrome-phenotype associations found in our study.

10. CONCLUSION AND FUTURE PERSPECTIVES

- Out of 11 investigated genetic susceptibility loci five showed correlation to specific morphologic features. The findings are consistent with pathogenic variants in these loci acting in distinct different CRC morphogenic pathways.

  - A 5 to 10 year follow-up of our patients could provide prognostic information in relation to the investigated SNPs. In case a correlation exists between some or all of the loci and prognosis, such information might in the future be used to select patients for intensified follow up or treatment.
  - Our data may be useful in understanding the basic tumorigenic pathways linking genetic changes and morphology in CRC. Immunostaining for selected markers could further elucidate these mechanisms.
  - Since allelic associations may be population specific, our genotype-phenotype correlations should be replicated in other populations.

- Tumor location is the factor having most influence on CRC morphology which is in line with tumors in different locations having different genetic and embryological backgrounds as well as developing in different physiological settings. Age is the most important determinant for the presence of multiple tumors and an important factor for the aggressiveness of the disease. The results could speak for different mechanisms of tumor development in young and old patients. Few morphological features are related to sex and only one to family history.

- Emergency CRCs in general show a more aggressive histopathological profile and more advanced stage than elective CRC. Our findings could speak for emergency CRCs being an inherently different group that may have a different etiological or genetic background.

  - A 5 to 10 year follow-up of our patients together with an immunohistochemical and genetic (SNPs) comparison of the tumors in relation to sex, age, location, family history and mode of presentation could indicate which proteins/ molecular pathways that differ in the carcinogenesis and if any of these can be used for prognostication.
  - In case a correlation is found between prognosis and some of the immunohistochemical markers studied, these markers could be included in routine pathology making it possible for the pathologist to contribute additional prognostic information in the individual case.
  - Even though the surgical aspects are important for the understanding of the worse prognosis in emergency CRCs, it is probably also of importance to characterize the biology of theses tumors since it might help us to design a more specific adjuvant treatment postoperatively.

- By using the family history of relatives to CRC patients we have identified five new putative CRC syndromes. Some supportive evidence of these was also found by genetic association and morphological analysis.

  - The concept of new CRC syndromes is intriguing and novel but our findings need to be replicated in further studies.

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