Colorectal Cancer

Aspects of Heredity, Prognosis and Tumour Markers

LANA GHANIPOUR
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**Abstract**


Colorectal cancer (CRC) is one of the most common cancer types and leading causes of cancer death worldwide. Since CRC is a heterogenic disease, there is a demand for increased knowledge of the underlying genetic and epigenetic mechanisms. The aim of this thesis was to investigate heredity and potential tumour markers in relation to prognosis. In paper I, survival of patients with CRC and a positive family history of CRC in first-degree relatives was analysed. Patients with colon cancer and positive family history of CRC had improved survival compared to patients with negative family history. This improvement in survival could not be explained by known clinico-pathological factors. In paper II, we investigated the prognostic value of Tryptophanyl t-RNA synthetase (TrpRS) in tissues from patients operated for CRC. Low protein expression of TrpRS in primary tumour tissues correlated with increased risk of recurrence and poorer survival. In paper III, the prognostic value of microsatellite instability (MSI) and the correlation to heredity for CRC in first-degree relatives was investigated. Patients with proximal colon cancer and MSI had improved cancer specific survival. There were no correlation between MSI and heredity. In paper IV, we evaluated the potential use of proximity ligation assay (SP-PLA) in patients with CRC, by simultaneous analysis of 35 proteins in only 5 μl plasma. SP-PLA is a suitable method for protein detection and might give valuable guidance in pursuing new prognostic and predictive tumour markers. However, none of the markers selected for present SP-PLA analyses gave better prognostic information than CEA. In conclusion, heredity is related to better survival independent of MSI in patients with CRC and MSI is associated with better prognosis in proximal colon cancer. Detection and increased knowledge of molecular mechanism in CRC is important, however it needs to be further investigated and validated in clinical use.

**Keywords:** colorectal cancer, heredity, Tryptophanyl t-RNA synthetase, microsatellite instability, SP-PLA, prognosis, biomarkers

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To my family
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals given below.


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Abbreviations

APC    Adenomatous Polyposis Coli
BAX    Bcl-2-Associated X protein
BRAF   v-raf murine sarcoma viral oncogene homolog
CEA    Carcinoembryonic antigen
CIMP   CpG Island Methylator Phenotype
CIMP-H CpG Island Methylator Phenotype- High
CIN    Chromosomal Instability
CRC    Colorectal Cancer
CSS    Cancer Specific Survival
DFS    Disease Free Survival
EGFR   Epidermal growth factor receptor
FA     Folinic Acid
FAP    Familial Adenomatous Polyposis
FU     Fluorouracil
HPA    Human Protein Atlas
HNPCC  Hereditary Non polyposis Colorectal Cancer
IHC    Immunohistochemistry
KRAS   v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
Lv     Leucovorin
LOH    Lost Of Heterozygosis
MLH1   MutL Homolog 1
MSH2   Mut S Homolog 2
MSH6   MutS Homolog 6
MSI    Microsatellite instability
MSI-H  Microsatellite instability -High
MSI-L  Microsatellite instability- Low
MSS    Microsatellite stable
MMR    Mismatch repair
OS     Overall survival
PCR    Polymerase Chain Reaction
PLA    Proximity ligation assay
PMS2   Postmeiotic segregation increased 2
RCT    Radiochemotherapy
SP-PLA Solid-phase proximity ligation assay
TMA    Tissue microarray
TME    Total Mesorectal Excision
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>TNM</td>
<td>Tumor Nodes Metastases</td>
</tr>
<tr>
<td>TrpRS</td>
<td>Tryptophanyl t-RNA synthetase</td>
</tr>
<tr>
<td>TTR</td>
<td>Time To Recurrence</td>
</tr>
</tbody>
</table>
Introduction

Colorectal cancer (CRC) is the third most common cancer and one of the leading causes of cancer-related deaths in the western world. In 2012 in Sweden, the incidence of CRC was approximately 6200 new cases, with colon cancer being the third most common cancer in both females (7.6%) and males (7.1%), after breast cancer, prostate cancer and skin cancer: while rectal cancer was ranked on seventh place and was overrepresented in males (1). Although the prevalence of CRC has slowly increased since the 1960s, the mortality rate has decreased over the past decades due to improved treatments (1). The lifetime risk of developing CRC is approximately 5%, and the risk increases with age. CRC is unusual under the age of 50 year. On average in Sweden, colon cancer has increased by 1% in women and 0.7% in men per year (1). Approximately two-thirds of all CRC cases are located in the colon and 1/3 in the rectum (Figure 1).

Figure 1. Distribution of cancer throughout the colon and rectum (1).
Aetiology

Heredity and environmental factors play an important role in the development of CRC. Up to 15% of all CRC are of heredity pattern, and family history is a strong risk factor for CRC development. Lifetime risk of developing CRC is double among those with first-degree relative having CRC, and the risk increases 4-fold if the diagnosis is set before the age of 45 years (2). The most known inherited CRC syndromes are Lynch syndrome, also called hereditary non-polyposis colorectal cancer (HNPCC), and familial adenomatous polyposis (FAP), both are autosomal dominantly inherited. Lynch syndrome accounts for 1-3% of all CRC cases and is caused by mutations in the DNA mismatch repair genes MLH1, MSH2, MSH6 and PMS2 (3). CRC occurs in Lynch syndrome at an average age of 45 years and an increased risk of malignant development in extra colonic organs may arise (3). FAP accounts for less than 1% of all CRC and occurs due to mutation of the adenomatous polyposis coli (APC) gene on chromosome 5 (4). The syndrome is characterised by early onset of multiple colorectal adenomas. Adenomas may occur in the upper gastrointestinal tract, and extra colonic organs can be affected. If untreated, one or more of the adenomas will become malignant by the median age of 40 year.

Polyps are a risk factor for the development of CRC; the risk increases with size and if they are histologically of villous type. Adenomas larger than 1 cm in size may have an approximately 8% risk of developing into a carcinoma over a 10-year period (5). The influence of dietary factors on the development of CRC remains controversial. The western lifestyle with low intake of fibre and vegetable or a diet of high saturated fat or red meat, processed meat, excessive alcohol consumption and obesity has been suggested as possible risk factors (6). Tobacco has been associated with large adenomas and is a factor for CRC risk (7). Factors with probably protective effect are intake of dietary fibre, fruit, vegetable, fish, calcium, physical activity and aspirin (8-10). Another group at high risk for developing CRC are patients with inflammatory bowel disease, ulcerative colitis and Crohn’s disease, and accounts for 1-2% of all CRC in the general population: and the risk increases further with early age at diagnosis of inflammatory bowel disease, duration of symptoms and severity of the inflammation (11).

Pathology and staging

CRC is histologically divided into several types, suggested by World Health Organisation (WHO), with adenocarcinoma, mucinous adenocarcinoma and signet ring cell cancer being the most common subtypes in decreasing order (Table 1). CRC is classified according to the tumour- lymph node - metastasis (TNM) staging system, which is most widely used and last re-
vised 2010 (12). This staging system provides information about the infiltrative growth of the primary tumour, spread to regional lymph node or distant organs (Table 2). The TNM-staging system provides broad prognostic information and facilitates decision-making in therapy (13). To predict the likelihood of detecting metastasis, at least 12 numbers of lymph nodes need to be examined, and the more lymph node that are examined by the pathologist is advantageous for survival in stage II CRC (14). The distance between tumour and transverse margin is considered optimal over 5 cm in order to avoid recurrence in the anastomosis.

Table 1. The classification of CRC subtypes suggested by WHO.

<table>
<thead>
<tr>
<th>Classification</th>
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<tbody>
<tr>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>Mucinous adenocarcinoma (&gt;50 % mucinous)</td>
</tr>
<tr>
<td>Signet-ring cell carcinoma (&gt;50 % signet-ring cells)</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>Adenosquamous carcinoma</td>
</tr>
<tr>
<td>Small cell carcinoma</td>
</tr>
<tr>
<td>Medullary carcinoma</td>
</tr>
<tr>
<td>Undifferentiated carcinoma</td>
</tr>
<tr>
<td>Other</td>
</tr>
</tbody>
</table>

There are several different tumour grading system based on architectural and/or cytological features, these describe the level of cell differentiation within the tumour, commonly through separation into four groups: well differentiated (grade 1); moderately differentiated (grade 2); poorly differentiated (grade 3); and, undifferentiated (grade 4) (13). Although, this grading system has been questioned, as it has not reached widespread acceptance (13), however high tumour grade is a prognostic unfavourable pathological factor, as are lymphatic- and vascular invasion by the tumour, absence of tumoral infiltrating lymphocytic response (15) and venous vessel invasion (13).
Table 2. TNM classification of CRC (seventh edition).

<table>
<thead>
<tr>
<th>Stage</th>
<th>T</th>
<th>N</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>T1-2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>II A</td>
<td>T3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>II B</td>
<td>T4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>III A</td>
<td>T1-2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>III B</td>
<td>T3-4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>IV</td>
<td>Any</td>
<td>N2</td>
<td>0</td>
</tr>
</tbody>
</table>

**T**
- **Tx**: Primary tumour cannot assessed
- **Tis**: Carcinoma in situ
- **T1**: Tumour invades submucosa
- **T2**: Tumour invades muscularis propria
- **T3**: Tumour invades through muscularis propria into subserosa or into non-peritonealized pericolonic or perirectal tissue
- **T4**: Tumour directly invades other organs or structures and/or perforates visceral peritoneum
- **T4a**: Perforates visceral peritoneum
- **T4b**: Directly invades other organ or structures

**N**
- **Nx**: Regional lymph nodes cannot be assessed
- **N0**: No regional lymph node metastasis
- **N1a**: Metastasis in 1 regional lymph node
- **N1b**: Metastasis in 2-3 regional lymph nodes
- **N1c**: Satellites in subserosa, without regional lymph nodes
- **N2**: Metastasis in 4 or more regional lymph nodes
- **N2a**: Metastasis in 4-6 lymph nodes
- **N2b**: Metastasis in 7 or more lymph nodes

**M**
- **M0**: No distant metastases
- **M1a**: Distant metastases in one organ
- **M1b**: Distant metastases in more than one organ or peritoneum

**Treatment**

The treatment of CRC should be individualised and discussed in a multidisciplinary team. Surgery is currently the primary treatment for patients with potentially curable CRC. Survival in patients with CRC has improved due to therapy implementation and curative resection is the strongest factor associ-
ated with survival. Radiation and chemotherapy, but also recently introduced targeted therapies have important place in the treatment of advanced CRC.

Surgery

The choice of surgical procedure for CRC is based on localisation and the expanse of the tumour. Following the embryonic planes, the tumour-bearing segment with its associated mesenterium, lymph nodes and lymph vessels are resected with a tumour free margin of at least 5 cm. In colon cancer surgery has complete mesocolic excision (CME) in the mesocolic plane and central vascular ligation and extensive resection of mesocolon with its lymphatic drainage and lymph nodes shown to have an impact on survival and recurrence (16-18). The procedure can be applied either by open surgery or laparoscopic. Laparoscopic surgery of CRC reaches the same long-term results as open surgery (19). The amount of resected lymph nodes is essential for correct staging of the tumour and choice of adjuvant therapy, and a minimum of 12 lymph nodes. The recommendation is need examining a minimum of 12 lymph nodes for correct staging of stage II CRC (20).

The surgical technique in rectal cancer with total mesorectal excision (TME) was introduced in the 1980s by RJ Heald (21). This technique involved the removal of the rectum together with mesorectum and the surrounding mesorectal fascia in order to reduce the local recurrence rate compared with previous surgical procedures (21). The TME technique is currently the golden standard in rectal cancer and provides a dissection outside the mesorectal fascia, with its corresponding lymph nodes and vessels surrounding the rectum, under direct vision. A clear circumferential margin, which is defined as a distance of >1 mm between the tumour tissue and the mesorectal fascia, is important, as a positive circumferential margin increases the risk of local recurrence rate and development of distant metastases (22). TME combined with radiation is the recommended standard treatment for rectal cancer, which has improved the local recurrence rates (23-25).

Colorectal liver metastasis is presented at initial diagnosis in approximately 25% of the patients and 25-35% will eventually develop metastasis during their disease (26). Patients with localised liver metastasis who undergoing radical surgical resection with curative intention are predicted a 5-year survival rate of 45% (27). The lung is the next most common site of distant metastasis in CRC and is seen in 10-20% of the patients (28). Patients undergoing complete resection of lung metastases are predicted a 5-years survival rate of between 24% and 56% (29, 30).

Radiotherapy

Radiotherapy is central in the treatment of rectal cancer. In advanced colon cancer, radiotherapy can be used as a palliative treatment. Several random-
ised trials have shown that radiotherapy given preoperatively or postoperatively in the treatment of rectal cancer decreases the risk of local recurrence (23, 25, 31). Preoperative radiochemotherapy (RCT) is, compared to postoperative given RCT, associated with lower local recurrence rate, reduced toxic adverse effects and increased sphincter preservation in low-lying rectal cancer (32). In Sweden, short-term high-dose preoperative radiotherapy is the standard treatment for patients with resectable rectal cancer with improved impact on reduction of local recurrence and survival (23).

In an attempt to adjust the treatment strategies, rectal cancer is classified according to the risk of local failure in categories as good, bad or ugly (33). Good rectal cancer (mid/upper rectum T1-3bN0 and low rectum T1-2N0, T3aN0) with intact mesorectal fascia, have a 5-year local recurrence rate of less than 10% (33), and because of the low recurrence rate only primary surgery is recommended. In the bad group (mid/upper rectum T3c/d N1-2, low rectum T3bN1-2 and T4 with peritoneal or vaginal involvement N1-2) without involvement of mesorectal fascia, the local recurrence rate is 10-20% (33), and short-course preoperative radiotherapy (5x5 Gy) followed by surgery is proposed. Ugly rectal cancer (T4 with overgrowth to surrounding organs, N1-2 and growth into circumferential marginal) has an increased 5-years local recurrence rate of 20-100% (33). In an attempt to reduce the tumour burden or down-staging, preoperative long-course radiotherapy alone or in combination with chemotherapy followed by delayed surgery is the preferred option (33).

Chemotherapy

Adjuvant chemotherapy

If the histopathological examination reveals lymph node metastasis, the tumour is classified as stage III, adjuvant chemotherapy is administered in an attempt to eliminate microscopic metastases and thereby reduce the risk of recurrence. Adjuvant chemotherapy is recommended for patients with stage III colon cancer, without any contraindications, as there is a recurrence rate between 15% and 50% (34). For stage II colon cancer, adjuvant treatment is still controversial: however high-risk groups for recurrence such as T4, acute operation, and with few lymph nodes analysed are offered treatment even though the benefits of that are not proven (35). In the 1990s, several studies showed that the administration of fluorouracil (FU) in combination with leucovorin (Lv) improved overall survival and disease free survival in patients with stage III colon cancer (35-37). The addition of oxaliplatin to FU/Lv (FOLFOX4) increases 5-year disease-free survival from 67.4% to 73.3%, and 6-year overall survival in stage III patients increased from 68.7% to 72.9% (38, 39) and reduces the risk of recurrence by 20% (39). Currently, the gold standard treatment is FU/Lv alone or in combination with oxalipla-
tion for 6 months for patients up to 75 years old (biological age) who might tolerate chemotherapy without any contraindications. In special cases oral fluoropyrimidine, capecitabine, is given as monotherapy or in combination with oxaliplatin (Xeloda), which has an equivalential affect as intravenous administered FU/Lv, and with a favourable toxicity profile (40).

Treatment strategies for rectal cancer differs from colon cancer. Patients with resectable rectal cancer and receiving preoperative RCT do not appear to benefit from postoperative adjuvant chemotherapy (41, 42).

**Palliative chemotherapy**

In an attempt to reduce tumour-related symptoms or prolong survival, palliative chemotherapy can be offered patients with metastatic CRC, and due to recurrence or advanced tumour stage, approximately 40% of the patients with CRC receive palliative treatment (43). FU/Lv in combination with either oxaliplatin or irinotecan prolongs progression-free survival and overall survival (43). Monoclonal antibodies for targeting cancer cells (bevacizumab, cetuximab) are used in recent years and have affects on overall survival and progression free survival only in combination with chemotherapy when used in advanced CRC. The choice of treatment should always be based on patient safety, with regard to drug toxicity and other side effects, and patient agreement.

**Prognosis**

Survival in CRC is stage dependent. In patients with CRC, 5-year survival is >90% for stage I, 60-85% for stage II, 45-65% for stage III and 5-7% for stage IV. At the time of diagnosis, about 20-25% of CRC patients have metastatic disease or will develop metastasis later (44). During the past decades, improvement in surgical skills and oncological treatment has influenced the impact on survival (45, 46). The median survival in patients with metastatic CRC has increased from 6 months up to 22-24 months in some of the cases, this due to oncological treatment and early detection of distant spread (43). Factors with poor impact on prognosis, including advanced stage, are obstruction/perforation, rupture during surgery, vascular and perineural invasion, poor differentiation, less than 12 lymph nodes analysed, and detection of molecular markers associated with poor prognosis (44). Preoperative elevated serum levels of carcinoembryonic antigen (CEA) are associated with increased risk of recurrence (47) and the CEA levels should be normalised within 4-6 week after curative intended surgery. Patients with high levels of early postoperative CEA have shorter time to recurrence with liver metastasis as a common site (48), and intensive follow-up examination for early detection of recurrence is recommended (48).
Between 8% and 34% of patients with colon cancer require emergency surgery, mostly due to obstruction or perforation (49, 50). This patient group undergoing emergency surgery is usually older; and has more advanced tumour stage with decreased survival rate, compared with those undergoing elective surgery (49). Recurrence for curatively treated patients with colorectal cancer can be distant, local, or both, and is related to the disease stage. The recurrence rate in colon cancer is 12.8% for local recurrence and 25.6% for distant recurrence (51). Prognostic factors affecting survival and recurrence rate in rectal cancer are tumour involvement of the mesorectal fascia and complete excision of mesorectum (52). Data from 1995 to 2001, in the Swedish Rectal Cancer Registry indicates a recurrence rate of 7.2% for patients in stage I rectal cancer who had local excision (53), seen in one study before the TME was used. The use of TME technique for rectal cancer treatment has led to local control and prolonged survival. Preoperatively short-term radiotherapy followed by surgery has led to improvement in local control and overall survival, with a local recurrence rate of 11%, compared to surgery alone (27%) (23). The 10-year cumulative incidence of local recurrence in patients treated with preoperative radiotherapy and TME is 5% compared to 11% for TME alone (54).

**Heredity**

Approximately 15% of all CRC arise due to genetic factors, and family history is one of the strongest risk factors for CRC development: lifetime risk of developing CRC is approximately 5%. The risk of CRC is increased among those patients with a family history of CRC, and the risk is even further increased if two or more relatives are affected and diagnosed at young age (2). This risk is particularly estimated from studies on first-degree relatives. Inherited CRC syndromes accounts of less than 5% of the cases and the most common inherited forms are Lynch syndrome and familial adenomatous polyposis, both of which are autosomal dominantly inherited.

**Lynch syndrome**

The term Lynch syndrome and HNPCC have historically been synonymous, and the definition of the syndrome has refined in recent years due to molecular genetic testing. Herein, the term Lynch syndrome will be used, which is today commonly accepted (55). The frequency of Lynch syndrome in Sweden accounts for 1 to 3% of all CRC (56). The syndrome is caused by mutation in DNA mismatch repair (MMR) genes MLH1, MSH2, MSH6 and PMS2, which causes inability to repair errors occurring during DNA replication, especially in the repeat sequences, microsatellites. This results in accumulation of mutations in coding and non-coding microsatellites, so-called
microsatellite instability (MSI). Mutations in MLH1 and MSH2 account for approximately 90% of all cases of Lynch syndrome CRC, while 7-10% of the mutations occurs in MSH6 and less than 5% in PMS2.

Identification of patients with Lynch syndrome is important because of its association with increased risk of cancer development: lifetime risk of developing CRC is 54-74% for men and 30-52% in women with Lynch syndrome (57). Mutations in DNA mismatch repair genes not only cause development of CRC but also an increased risk of extracolonic cancer. Cancer of endometrium is the second most common site. Lifetime risk of endometrial cancer development is 2-3% in the general population, and 28-60% in patients with Lynch syndrome (57).

Many criteria have been proposed for identifying patients with Lynch syndrome, and are mostly based on age at diagnosis, presence of multiple tumours and number of affected families. The syndrome is clinically characterised through the revised Bethesda guidelines for selecting patients for further genetic analysis (59). The classic technique for MSI mutation analysis is by polymerase chain reaction (PCR). The national cancer institute has recommended a panel of five specific microsatellite markers for detection, the Bethesda panel. If two or more of the microsatellite markers show instability, the tumour is termed as MSI-high (MSI-H), and if only one marker is unstable, the tumour is referred as MSI-low (MSI-L). Tumours that show no instability are called microsatellite stable (MSS) (60). Another accepted method for detecting MMR mutations is by immunohistochemistry (IHC). Detecting inactivated MMR proteins by IHC, has shown high sensitivity and specificity (61-64). However, approximately 10% of patients with Lynch syndrome will express positive staining with IHC (57). Further mutation analysis is offered to patients detected by IHC as having MSI. Analysis of MSI with PCR has higher sensitivity than IHC, and is able to identify defective MMR genes that IHC does not detect (65), but the former method is time-consuming and expensive.

Characteristics for Lynch syndrome tumours are proximal colonic location, mucinous or signet ring cell type, poor differentiation, and the presence of infiltrating lymphocytes (57). The main clinical feature is early age of diagnosis and the occurrence of multiple tumours. The average age at diagnosis of CRC in Lynch syndrome is between 42 and 61 years, which is lower than the general population (57). Periodic examination by colonoscopy is recommended for detecting CRC in an early stage. A 63% risk reduction in CRC development can be achieved with periodic colonoscopy and thus, reducing the cancer related mortality (58). The optimal interval for colonoscopy for mutation carriers is recommended between 1 and 2 years (66). Prophylactic colectomy in the absence of CRC diagnosis is considered restricted, however, if the patient is unwilling or incapable of undergoing periodic colonoscopy, it could be a consideration (57).
Table 3. Criteria for identifying patients with Lynch syndrome

<table>
<thead>
<tr>
<th>Amsterdam II criteria (67)</th>
<th>Revised Bethesda guidelines (59)</th>
</tr>
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<tbody>
<tr>
<td>1. At least three relatives with CRC or a Lynch syndrome-associated cancer (endometrium, small bowel, ureter or renal pelvis) and,</td>
<td>Tumours from individuals should be tested for MSI in the following situations:</td>
</tr>
<tr>
<td>2. One first-degree relative of the other two, and</td>
<td>1. CRC diagnosed in a patient &lt;50 years of age.</td>
</tr>
<tr>
<td>3. At least two affected generations, and</td>
<td>2. Presence of synchronous, metachronous CRC, or other Lynch syndrome associated tumours, regardless of age.</td>
</tr>
<tr>
<td>4. One cancer diagnosed before the age of 50 years, and</td>
<td>3. CRC with MSI-H histology diagnosed in a patient who is &lt;60 years of age.</td>
</tr>
<tr>
<td>5. FAP excluded in the CRC case.</td>
<td>4. CRC diagnosed in one or more first-degree relatives with a Lynch syndrome- related tumour, with one of the cancers being diagnosed under age 50 years.</td>
</tr>
<tr>
<td><strong>All criteria must be fulfilled for further analyses.</strong></td>
<td>5. CRC diagnosed in two or more first- or second-degree relatives with Lynch syndrome -related tumours, regardless of age</td>
</tr>
</tbody>
</table>

### Familial adenomatous polyposis

Familial adenomatous polyposis (FAP) is caused by mutation in the tumour suppressor gene of APC, resulting in multiple adenomatous colorectal polyps. The APC gene is detected in 80-90% of patients with this syndrome. FAP accounts for approximately 1% of all CRC cases. In the absence of treatment, the risk of developing CRC is 100% by age 40 (68). Polyps in the upper gastrointestinal tract are present in nearly 90% of patients with FAP (69). CRC and duodenal cancer are the most common cause of death in FAP patients. The lifetime risk of developing duodenal cancer is approximately 4% in patients with FAP, which also is the second cause of death (70). Other malignancies associated with FAP are papillary thyroid cancer, medulloblastomas, hepatoblastomas in children, desmoids tumours (68). Genetic testing is routinely used for detection of FAP. Flexible sigmoidoscopy at the age of 10-12 years old is recommended for screening for polyps in APC gene mutation carriers (68). Once polyps are detected, annual colonoscopy for polyp screening is recommended and when the polyp burden increases, prophylac-
tic colectomy is offered. If the rectum is left, annual endoscopy is needed because of the risk of adenoma development. Screening for polyps and adenomas in the upper gastrointestinal tract with gastroduodenoscopy is preferred to initiate at the age of 25-30 years, every 1-3 years depending on the poly burden (71).

Other inherited syndromes with risk of CRC malignancy are: MUTYH-associated polyposis, juvenile polyposis and Peutz-Jeghers syndrome.

Familial colorectal cancer
A positive family history of CRC is defined as the presence of CRC in one or more first- and/or second-degree relatives. Familial CRC is defined as a history of two or more cases of CRC within the family where known hereditary syndromes are excluded, and accounts for approximately 20-25% of all CRC cases in Sweden (72). The lifetime risk of developing CRC is 5%, but this risk doubles when at least one first-degree relative is diagnosed as having CRC, and even further increases when at least two first-degree relatives are affected or if the affected relative is below the age of 50 years (73).

Pathways of tumourogenesis in colorectal cancer
CRC arises due to the accumulation of genetic and epigenetic alterations from normal colonic epithelium to carcinoma. In the year 1990, Fearon and Vogelstein were first to describe a genetic model for colorectal tumorigenesis through several steps leading to accumulation of mutations in tumour suppressor genes and oncogenes from a normal cell to carcinoma (74). In this process, four to five genetic alterations are required, and this multistep event may take several years to cancer development. Tumorigenesis in sporadic CRC is described arisen from different pathways: the most known are chromosomal instability (CIN) and microsatellite instability (MSI) pathway. Recently a third pathway, CpG island methylator phenotype (CIMP), mediated through promoter methylation of tumour suppressor genes, has been described (75, 76).

Chromosomal instability pathway
Chromosomal instability (CIN) is the most common pathway of genomic instability, and occurs in approximately 60-70% of all CRC cases. CIN is characterised by accumulation of mutation in tumour suppressor genes and oncogenes, such as APC, TP53, SMAD2, SMAD4, DCC, KRAS, PIK3CA, and loss of heterozygosity (LOH), which is commonly found in chromosome
1, 5, 8, 17 and 18 (77). These events lead to transformation of normal colonic epithelium to colon adenocarcinoma. A majority of MSS tumours follows the CIN pathway. Characteristically for CIN tumours are that they are often aneuploid or polypoid, highly differentiated, rarely mucinous, have no lymphocytic infiltration, poor prognosis, and no specific tumour site predominance (78).

Microsatellite instability pathway
Microsatellite instability (MSI) occurs in approximately 15% of sporadic CRC and more than 95% of all Lynch syndrome cases. MSI in sporadic CRC is predominantly caused by hypermethylation of the promoter region of MLH1, resulting in transcriptional silencing (79). MSI in Lynch syndrome arises due to germline mutations of one of the DNA MMR genes MLH1, MSH2, MSH6 and PMS2. In both way, MSI results in inactivation of the MMR system, and thereby failure to correct nucleotide mismatches, and causes a high frequency of mutations in coding and non-coding regions of repetitive sequences throughout the cancer genome. Typical for MSI tumours are frameshift mutations in specific genes such as β-catenin, transforming growth factor β receptor II (TGFβRII), epidermal growth factor receptor (EGFR) or Bel-2-associated X protein (BAX) (80-82). It is known that MSI tumours display distinct clinico-pathological features such as proximal colonic site, mucinous or signet ring cell type, poor differentiation, presence of infiltrating lymphocytes, diploid phenotype, and fewer KRAS and p53 mutations (78). MSI tumours are also associated with larger tumour size and more favourable stage (78). Another common finding is that they are more frequently seen in women (78). Rectal tumours exhibiting MSI are rare, in cases of occurrence; they are often associated with Lynch syndrome (83). MSI tumours are categorised as MSI-H, MSI-L, and MSS according to the Bethesda panel. MSI-L tumours have the same clinicopathological features as MSS tumours, both linked to the CIN pathway and are associated with poorer survival than MSI-H tumours (78).

CpG island methylator phenotype pathway
The promoter of approximately 50% of all genes contains CpG islands (84). Promoter CpG island hypermethylation results in inactivation of tumour suppressor and DNA repair genes causing transcriptional silencing, which reflects an epigenic change (85). Extensively hypermethylated tumours are classified as CIMP + or CIMP-high (CIMP-H) in the literature. The MLH1 gene is frequently inactivated in this epigenetic event and is found in approximately 35-40% of all CRCs, and has also been identified in adenomas (86). CIMP-H tumours are associated with proximal colon localisation, older age, MSI, high frequency of BRAF and KRAS mutations and poor differentiation
Although these characteristics of CIMP-H tumours reflect the clinicopathological features of MSI-H tumours, CIMP-H in MSS tumours are associated with lymph node and liver metastasis and poor prognosis (85). This unfavourable effect on the outcome of CIMP-H MSS tumours might be due to the presence of KRAS/BRAF mutation (85).

**Prognostic and predictive tumour markers**

CRC is one of the leading causes of death in the western world. The traditional pathological staging system for predicting the outcome is limited because of the heterogeneous biology of the disease. Predictive molecular markers are desirable for selecting an optimal, personalised treatment strategy for the patient. The role of genetic and epigenetic alternations is important, especially for identification and application of prognostic- and predictive markers in routine clinical use, but also to overcoming the toxicity and medical cost it entails.

**Carcinoembryonic antigen**

In 1965, Gold and Freedman, identified carcinoembryonic antigen (CEA) in malignant tumours of the entodermally derived epithelium from the gastrointestinal tract and pancreas (88), which appeared to be absent from healthy colon. CEA belongs to the immunoglobulin superfamily and is attached to the cell membrane by glycosyl phosphatidylinositol anchor and released in soluble form by phospholipase C or D (89, 90). CEA is today the most important and commonly used serum tumour marker in clinical practice and is recommended for determining prognosis, surveillance followed after curative resection, and as a monitoring therapy in advanced CRC (91, 92). However, because of the low sensitivity (18-69%), early detection of CEA in CRC is not recommended (93). The factors affecting serum levels of CEA in patients with CRC are: disease stage, differentiation grade, liver disease, tumour site, bowel obstruction, smoking and ploidy status of tumour (90). A high level of CEA, taken preoperatively, is associated with adverse prognosis in patients with CRC, and these patients may not benefit from adjuvant chemotherapy based on the increased level of CEA (90). Increased postoperative CEA is associated with early recurrence (48).

**APC**

Adenomatous polyposis coli (APC) is the most common mutated gene in familial adenomatous polyposis (FAP) and is present in 70-80% of all sporadic colorectal adenomas and carcinomas (84). APC is a key regulator of the wnt-signaling pathway. Inactivation of APC gene is the initial genetic
event in adenoma-carcinoma sequence and leads to accumulation of β-catenin in the cytoplasm and nucleus, leading to activation of wnt-signalling pathway, resulting in transcription of downstream target genes (94). APC has no role in prognosis and therapy settings.

**KRAS**

KRAS is a protooncogen and a member of the RAS gene family. Mutations in KRAS results in constitutive EGFR independent activation of increased tumour cell proliferation, protection against apoptosis, giving cells the ability to metastasise, and stimulating angiogenesis (95). KRAS mutations are early events in CRC tumorigenesis and occur in 30-50% of all CRC cases (96-98). Single nucleotide point mutations are more commonly occurred in codons 12 and 13 of exon 2, and to a lesser extent in codon 61 of codon 3 (77). KRAS mutations are more commonly seen in MSI-L and MSS tumours. The prognostic relevance of KRAS mutations is controversial. The majority of the studies associate KRAS mutations with poor prognosis (99, 100). Some associates its prognostic implications to stage of disease (101), specific mutation site or in combination with mutations in p53 (102, 103). Recently the focus on KRAS mutation status has shifted towards being a predictive marker in metastatic CRC with anti-EGFR target therapy (104). As KRAS gene mutation is associated with a negative response to anti-EGFR target therapy (105-107), patients with metastatic CRC and KRAS mutation in codon 12 or codon 13 are not recommended anti-EGFR therapy (108). Testing for KRAS mutation in patients with metastatic CRC before initiation of anti-EGFR therapy has been implicated in clinical practise (109).

**BRAF**

BRAF is an oncogene, a member of the RAS family, and a mediator of the EGFR pathway. Mutations in BRAF occur at early stage of colorectal carcinogenesis, and lead to constitutive activation and deregulation of the downstream signalling pathways (110). BRAF mutations are found in 4-15% of all sporadic CRC cases, of which 34-70% are seen in MSI-H tumours by epigenetic inactivation of the MMR system, usually by promoter hyper-methylation of MLH1 and not due to germline mutations as in Lynch syndrome (68, 111): only 1.4% of patient with Lynch syndrome carry a BRAF mutation (112). BRAF mutations are more frequently found in right-sided tumours and in tumours of poorly differentiated adenocarcinoma or mucinous carcinoma (113). More than 90% of all BRAF mutations involve V600E, by a substitution of valine-to-glutamic acid amino acid, resulting in abnormal activation of MEK-ERK pathway (114). Mutations in BRAF have been associated with poor clinical outcome (115). Patients with MSS tumours and BRAF mutations have shorter progression-free survival and overall survival.
Patients with BRAF V600E mutations are shown to have decreased benefit of EGFR-targeted monoclonal antibody therapies (118), however, the predictive role of BRAF can be of useful information in the selection of patients with metastatic CRC current for EGFR inhibitor therapy.

18q LOH

Allelic loss of chromosome 18q, causing tumour suppressor gene inactivation, occurs frequently in CRC and often late in the carcinogenic event (119, 120). Tumour suppressor genes located in 18q includes DCC, SMAD2, SMAD4 and CDK5 (121). CRC with loss of 18q are associated with adverse clinical outcome, but the prognostic importance of 18q LOH is of inconsistent results with heterogeneous detection methods (122); in some studies, 18q LOH is associated with poor survival (123, 124), whereas, in other studies is not (125, 126). The prognostic role of 18q LOH may be a substitute marker for the CIN pathway rather than an independent prognostic marker.

p53

As a tumour suppressor gene, p53 is involved in cell cycle inhibition, apoptosis, genetic stability and inhibition of angiogenesis (127). Mutations in the p53 gene appear late in the adenoma-carcinoma sequence and are found in approximately 40-50% of all CRC cases (128). Mutations in the p53 gene are more commonly seen in tumours with distal colon location, high stage differential grade and MSS tumours (129). There is controversial meaning about the relationship of p53 mutations and prognosis in CRC. Mutations of p53 in proximal tumours and the p53 G245 hot spot mutations are associated with poorer prognosis (130). The determination of p53 status for evaluating the prognosis or predicting response to chemotherapy in clinical practise is not routinely recommended (92).

MSI

As mentioned above, MSI reflects the presence of defective mismatch repair genes, and tumours that displaying MSI have more distinct clinicopathological features than MSS tumours. The prognostic significant of MSI tumours is their association with favourable outcome (131). Mutations in TGF-βRII, BAX or EGFR are often seen in tumours exhibiting MSI-H (82), however, mutations in these genes have not shown a significant impact on the favourable prognosis characterising MSI colon tumours (132). Unlike Lynch syndrome, mutation in BRAF V600E gene is commonly occurred in sporadic MSI tumours (133, 134). There is an on-going debate regarding MSI and the responsiveness to 5-FU chemotherapy, since studies have shown that MSI-H tumour cells are resistant to 5-FU (135). It is believed that this resistance...
may be due to MLH1 hypermethylation, as the cells regain their sensitivity towards 5-FU upon MLH1 demethylation (136). Patients with MSI-H colon cancer stage II and III do not have the same survival benefit from adjuvant 5-FU chemotherapy as patients with MSS and MSI-L tumours (137, 138). Although studies have shown that MSI-H colon cancer does not benefit from adjuvant 5-FU, the current standard adjuvant treatment for stage III is still FU/Lv and oxaliplatin regardless MSI status.

Human Protein Atlas

The Swedish Human Protein Atlas project (HPA) allows a systematic investigation of the human proteome (139). Through the use of antibody-based proteomics, HPA aims to produce a comprehensive atlas of protein expression pattern in 46 different human normal tissues, 20 most common cancerous tissues, and 47 different human cell lines, assembled in tissue microarrays (139, 140). The main purpose of the HPA project is to produce specific antibodies to all non-redundant proteins with a high-throughput method involving the cloning and expression of protein epitope signature tags and to create a map of human protein expression of these antibodies (141). The HPA project can be used as a tool for in silico biomarker discovery (142, 143). To date, approximately 82% of human protein-coding genes have been released.
CRC is a heterogeneous disease with various kinds of molecular and clinico-pathological characteristics. Prognostic markers are desirable in order to predict patient outcome and therapy implication. The overall aim of this thesis was to investigate relevant prognostic tumour markers in CRC and their correlation to heredity and prognosis.

Specific aims:

• To analyse survival of patients with CRC and a positive family history for CRC in first-degree relatives compared with those with no family history, and to determine whether differences in survival could be explained by clinico-pathological factors (Paper I).

• To evaluate the prognostic value of Tryptophanyl-tRNA synthetase in CRC (Paper II).

• To evaluate the correlations between defect MMR, prognosis, and heredity for CRC in first-degree relatives (Paper III).

• To explore the potential use of SP-PLA in patients with CRC (Paper IV).
Material and methods

Patients

The studies included in this thesis are based on one prospective population-based cohort.

Papers I-IV

Patients operated for CRC were consecutive included in a population-based cohort of 320 patients during the time period August 2000 and December 2003 at the Central district Hospital in Västerås, Sweden. Information on tumour size, lymph node status, lymphatic or vascular vessel invasion, mucinous cell type and tumour differentiation were retrieved from pathological records. Information about family history was obtained preoperatively, through a written questionnaire. Information on clinical stage, cancer recurrence, death, and causes of deaths were obtained from the Swedish Colorectal Cancer Registry (SCRCR) and surgical and oncological hospital records. In the study (Paper IV), 60 of the 320 patients were strategically selected, and divided into two groups: those with disease dissemination (stages I-III with recurrence and stage IV), and those with non-dissemination (stages I-III without recurrence).

Methods

Paper I

The patients (n=320) were asked preoperatively about family history and a written questionnaire was collected. Only patients with first-degree relatives (parent/sibling/offspring) with CRC were included. The main reasons for not participating in the study were acute operation, patients unable to answer heredity questionnaire, non-admission to the hospital due to palliative care, and cancer in a polyp. Information on first-degree relative with CRC was confirmed by histopathology reports. Patients with suspect Lynch syndrome were identified according to Amsterdam II criteria (Table 3) and they were also excluded from the study, as these patients represent CRC subtype with known favourable prognosis. The patients were followed until death or at the
end of the study period (1\textsuperscript{st} November 2008). The median follow-up time was six years (range 4-7) years.

Common methods Papers II-III

**Tissue microarray construction**

All cases were histopathologically re-evaluated on hematoxylin and eosin stained sections by one pathologist, and for each case, two 1.0-mm cores from the invasive tumor and one 1.0-mm core from normal mucosa, adenomatous mucosa, and lymph node metastasis were taken with a manual arraying devise (MTA-1, Beecher Instruments) for construction of tissue microarray (TMA).

**Immunohistochemistry and annotation**

Tryptophanyl t-RNA synthetase (TrpRS) has been identified as a prognostic marker by HPA. The protein expression of TrpRS in normal colonic and rectal mucosa is negative to weak (Paper II). Normal colon and rectum epithelium has strong MLH1, MSH2 and MSH6 protein expression located in the nuclei (Paper III).

Immunohistochemistry (IHC) was performed on 4-µm TMA sections using a polyclonal, monospecific IgG TrpRS antibody (WARS), generated by HPA (Paper II). Monoclonal antibody against MLH1 (Clone ES05, art.nr M3640, Dako), MSH2 (Clone FE11, art.nr NA27, Calbiochem (VWR)) and MSH6 (Clone EPR3945, art.nr 2846-B, Epitomics (Biosite)) were used (Paper III). The IHC on TMA sections were scanned in high-resolution scanners (ScanScope T2, Aperio Technologies) and separated into individual spot images representing the different cores in the TMAs. The spot images were evaluated with a web-based annotation system (i.e., imagescope viewer, a digital pathology system) (Papers II-III). Annotation of spot images included intensity of immunoreactivity for TrpRS [negative (0), weak (1), moderate (2), or strong (3)], and fraction (%) of TrpRS positive cells [<1% (0), 1-25% (1), 25-75% (2), or >75% (3)]. A four-graded scale was created for intensity and fraction of TrpRS score (Paper II).

Complete absent of nuclei staining of MLH1, MSH2 or MSH6 was regarded as defect MMR and documented as MSI. Tumour cells expressed with positive nuclei staining were considered as MSS (Paper III).

Additional methods Paper III

**MSI mutation analysis**

MSI analysis was performed in 32 of the invasive tumour cases by MSI analysis system, version 1.2 (Promega, Madison, WI) with 6ng genomic DNA, using the Bethesda panel of microsatellite markers (BAT25, BAT26,
Analyses were performed on a 3130 x 1 genetic analyser (Applied Biosystems, Foster city, CA) MSI-H was stated when 2 or more microsatellite markers showed instability: and if only one marker showed instability the tumour was defined as MSI-L. If no marker displayed instability, the tumour was stated as MSS. In the interpretation of the MSI analyses, MSI-L was grouped together with MSS.

**KRAS and BRAF mutation analysis**

For the pyrosequencing assay, PyroMark Q24 (Qiagen GmbH, Hilden, Germany) was used on fresh frozen tumour tissue samples from 207 patients for analysis of KRAS mutation and from 32 patients for BRAF mutation analysis. In brief, genomic DNA was extracted from fresh frozen tumour tissue and amplified by quantitative PCR (Stepone, Applied Biosystems Inc., Foster City CA) using KRAS and BRAF mutation QIAamp Mini Kit (Qiagen GmbH, Hilden, Germany). KRAS mutation of codon 12,13 and 61, and BRAF mutation of codon 600 were analysed.

**Paper IV**

**SP-PLA**

For protein detection with multiplex SP-PLA, 5 µl plasma from each patient was used, as described by Darmanis (144). The multiplex protein detection panel, comprising of 35 proteins previously reported as biomarkers for cancer, inflammation and cardiovascular disease, and one internal control (mouse IgG), was preselected by the science group of U Landegren for explorative studies. Prior to analysis, molar protein concentrations per 5 µl were converted to pM/µl.
Statistical analyses

Papers I-IV

Chi-square test was used for comparing dichotomous variables and for testing differences in proportions between groups (Papers I-IV). The Mann-Whitney U test was used to compare two nonparametric groups, and the Kruskal-Wallis test for comparison of more than two nonparametric groups (Papers I-II, IV). Continuous variables were given as median (range) (Papers I, IV). Cox proportional-hazard ratio for univariate and multivariate analyses was used to assess the effect of clinic-pathological variables on survival, including family history (Paper I), TrpRS score (Paper II) and MSI status (Paper III). The calculated hazard ratio was used as the RR estimate. A p-value <0.05 was considered as statistically significant. Kaplan-Meier curves were plotted graphically to illustrate survival, and were tested for significance with the log-rank method (Papers I-III). Overall survival (OS) was measured from the date of surgery to the date of death due to any cause. Disease-free survival (DFS) was measured for curatively operated patients, from the date of surgery to the date of diagnosis of a local/distal recurrence, second primary CRC/non-CRC, and the date of death due to any cause. Time to recurrence (TTR) was calculated in curatively treated patients from the date of surgery to the date of locoregional/distant metastasis recurrence, or to the date of death due to CRC. Cancer specific survival was measured from the date of surgery to the date of death in CRC.

All observations were censored at the end of the study period in 1st November 2008 (Papers I-II), and in 15th April 2010 (Papers III-IV). Statistical analyses were made with STATISTICA software (version 8.0) (Papers I-II) and SPSS version 21 (SPSS Inc., Chicago, Illinois, USA) (Papers III-IV).
Ethics

Papers I-III
Ethical approval (no. 00-001) was obtained from the Ethics committee at Uppsala University, Uppsala, Sweden.

Paper IV
Ethical approval (no. 2000:001) was obtained from the Ethics committee at Uppsala University, Uppsala, Sweden.
Results

Paper I
Of 318 treated for CRC, 37 patients had a history of first-degree relative with CRC. After excluding patients with non-verified histopathological report (4 patients) and Lynch syndrome (based on Amsterdam II criteria; 2 patients), thirty-one patients remained included in the study. None of the patients with positive family history was diagnosed as part of a symptom-free screening due to family history of CRC. Patients with positive family history had a lower T (tumour) stage (p=0.008) and were more able to develop second primary cancer, such as kidney, prostate, urinary bladder, skin, lung and breast cancer (p<0.001) than patients with no family history. In those patients with a positive family history and colon cancer, there was improved overall survival (p=0.012) (Figure 2), but this was not the case for patients with rectal cancer (p=0.416). No recurrences were observed in patients curatively treated for colon cancer and positive family history of CRC (p=0.035) (Figure 3). In the multivariate analysis, there was an increased risk for shorter overall survival among patients with no family history for CRC.

![Figure 2. Overall survival in patients treated for colon cancer with positive and negative family history for colorectal cancer in first-degree relatives.](image)
Figure 3. Time to recurrence in curatively treated patients with colon cancer and positive or negative family history of colorectal cancer in first-degree relatives.

**Paper II**

The expression of TrpRS staining was present in the cytoplasm of both cancer and normal cells (Figure 4). The fraction and intensity of TrpRS was higher in lymph node metastasis and invasive tumour tissue than in normal tissue. There was a correlation between low intensity of TrpRS and increased risk of lymph node metastasis (p=0.025) and advanced tumour stage (p=0.001). A higher TrpRS score was correlated with right colon localisation (p=0.001), higher age (p=0.040) and lower tumour stage (p<0.001). Patients curatively treated for CRC stage I-III (Figure 5a), and when divided separately for colon cancer stage I-III (Figure 5b), with TrpRS score 3, had longer time to recurrence than with TrpRS score 1 and 2. An improved disease-free survival was seen in a multivariate analysis among patients with CRC and TrpRS score 3 than for patients with TrpRS score 1 and 2 (RR, 0.59; 95% CI, 0.38-0.95). For patients with colon cancer and increased expression of TrpRS, there was a reduced risk of recurrence (RR, 0.23; 95% CI, 0.07-0.80).
Figure 4. Tryptophanyl-tRNA synthetase (TrpRS) expression on tissue microarray (TMA) from primary tumour tissue in patients with invasive colon cancer. The positive staining is mainly seen in the cytoplasm as a brownish colour; 4a-d: higher resolution showing negative (A), weak (B), moderate (C) and strong (D) staining intensity.
Figure 5. Time to recurrence in curatively treated patients with different immunohistochemical expression of tryptophanyl-tRNA synthetase (TrpRS) in invasive primary tumour tissue. TrpRS-score 1-2 represent low to medium expression and TrpRS-score 3 represent high expression. 5a: Colorectal; stages I-III; 5b: Colon; stages I-III.

Paper III

From 320 patients with CRC, forty-seven (15%) had negative expression of MLH1, MSH2 or MSH (Table 4, Figure 6). Clinico-pathological characteristics for MSI tumours were proximal colon localisation (p<0.001), mucinous
cell type \( (p=0.010) \), female gender \( (p=0.030) \), large tumour size \( (p < 0.001) \), poor differentiation grade \( (p=0.020) \), age above 75 years \( (p=0.010) \), and favourable disease stage \( (p=0.003) \) (Table 5). Patients with proximal colon cancer exhibiting MSI had improved cancer specific survival \( (p=0.006) \) (Figure 7a) and prolonged time to recurrence \( (p=0.040) \) (Figure 7b). No cases of MSI were identified in patients with rectal cancer and there were no recurrences in eight of the patients in stages II-III distal colon cancer exhibiting MSI. No correlation between MSI and heredity could be detected.

Table 4. Amount of intact and defect MMR protein in patients with CRC by IHC expression in invasive tumour.

<table>
<thead>
<tr>
<th></th>
<th>MLH1</th>
<th>MSH2</th>
<th>MSH6</th>
<th>MLH1MSH2MSH6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defect</td>
<td>38</td>
<td>6</td>
<td>8</td>
<td>47</td>
</tr>
<tr>
<td>Intact</td>
<td>276</td>
<td>313</td>
<td>309</td>
<td>268</td>
</tr>
<tr>
<td>Not representative</td>
<td>6</td>
<td>1</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>

Figure 6a-c. Positive (left) and negative (right) protein expression of (a) MLH1, (b) MSH2, (c) MSH6 detected with immunohistochemistry in primary tumour tissue from patients with colorectal cancer.
Table 5. Table 2. Comparison of clinicopathological characteristic in MSI and MSS colorectal cancer patients, analysed with IHC on tissue microarray from the primary tumour.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Defect MMR Primary cancer</th>
<th>Intact MMR Primary cancer</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of patients (%)</td>
<td>47 (15%)</td>
<td>268 (84%)</td>
<td></td>
</tr>
<tr>
<td>Tumour localization</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Right colon (%)</td>
<td>39 (83%)</td>
<td>83 (31%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Left colon (%)</td>
<td>8 (17%)</td>
<td>79 (29%)</td>
<td></td>
</tr>
<tr>
<td>Rectum (%)</td>
<td>0</td>
<td>106 (40%)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>30 (64%)</td>
<td>125 (47%)</td>
<td>0.030</td>
</tr>
<tr>
<td>Male</td>
<td>17</td>
<td>143</td>
<td></td>
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<tr>
<td>Age</td>
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<td></td>
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</tr>
<tr>
<td>&lt;75</td>
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<td>162</td>
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<tr>
<td>≥75</td>
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<td>106</td>
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<td>Family history</td>
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<tr>
<td>No</td>
<td>40</td>
<td>228</td>
<td>1.000</td>
</tr>
<tr>
<td>First degree relative with CRC</td>
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</tr>
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<td>Distant metastases (M)</td>
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<td>1</td>
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<td>40</td>
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</tr>
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<tr>
<td>Tumour size</td>
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<td>&lt; 4 cm</td>
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<td>126</td>
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<tr>
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<td>2</td>
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<tr>
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<td>217</td>
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</tr>
<tr>
<td>Poor</td>
<td>16</td>
<td>51</td>
<td></td>
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<tr>
<td>Vascular invasion</td>
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</tr>
<tr>
<td>No</td>
<td>46</td>
<td>229</td>
<td>0.0016</td>
</tr>
<tr>
<td>Yes</td>
<td>1</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>Mucinous cell type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>35</td>
<td>237</td>
<td>0.010</td>
</tr>
<tr>
<td>Yes</td>
<td>12</td>
<td>31</td>
<td></td>
</tr>
</tbody>
</table>

*loss of MLH1, MSH2 or MSH6 protein expression in invasive tumour tissue
Figure 7. Cancer specific survival (a) and time to recurrence (b) among patients with proximal colon cancer with respect to their MSI status, analysed by Kaplan-Meier curve.

Paper IV
In 60 patients, 31 had disseminated disease and 29 were without dissemination. A total of 21 of 35 proteins were detectable with SP-PLA (Table 6). Patients with disease dissemination had a median age of 69 (range 34-85) years and patients without disease dissemination had a median age of 76 (range 49-91) years. There were no statistically significant difference in gender, age, tumour localisation, and vascular- and neural invasion between patients with disease dissemination and non-dissemination. The plasma concentration of CEA was higher in patients with disease dissemination than in...
those without disease dissemination (p=0.007), which could also be seen in patients with advance disease stages (p=0.040) (Table 7). Patients with disseminated disease stage II-III had lower plasma levels of HCC-4 (p=0.003) (Figure 8). Low plasma concentrations of TIMP-1 were seen in patients with disseminated disease stage II (p=0.003) (Figure 9). There was no difference in plasma level of detectable proteins and disease stages I-IV CRC.

Table 6. Detectable and non-detectable proteins involved in different pathways.

<table>
<thead>
<tr>
<th>Pathways</th>
<th>Detectable (n=21)</th>
<th>Non-detectable (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Markers for inflammation</td>
<td>IL8</td>
<td>IL7</td>
</tr>
<tr>
<td></td>
<td>IL17a</td>
<td>IL6</td>
</tr>
<tr>
<td></td>
<td>VEGF</td>
<td>IL4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL1α</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TNF-α</td>
</tr>
<tr>
<td>Markers for cardiovascular disease</td>
<td>GDF-15</td>
<td>CD40 ligand</td>
</tr>
<tr>
<td></td>
<td>ICAM-1</td>
<td>Cystatin B</td>
</tr>
<tr>
<td></td>
<td>P-selectin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E-selectin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCL2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cystatin C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CF3</td>
<td></td>
</tr>
<tr>
<td>Markers for cancer</td>
<td>CXCL5</td>
<td>EGF</td>
</tr>
<tr>
<td></td>
<td>Kallikrein 6</td>
<td>GH</td>
</tr>
<tr>
<td></td>
<td>HCC-4</td>
<td>Artemin</td>
</tr>
<tr>
<td></td>
<td>TIMP-1</td>
<td>p53</td>
</tr>
<tr>
<td></td>
<td>TIMP-4</td>
<td>PSA</td>
</tr>
<tr>
<td></td>
<td>Follistatin</td>
<td>NGF-β</td>
</tr>
<tr>
<td></td>
<td>CCL4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCL5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cathepsin B</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cathepsin S</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fas</td>
<td></td>
</tr>
<tr>
<td>Internal control</td>
<td></td>
<td>Mouse IgG</td>
</tr>
</tbody>
</table>

Table 7. Comparison of plasma concentration of CEA between the disease stages in patients with CRC.

<table>
<thead>
<tr>
<th>Disease stage</th>
<th>CEA &lt;6ng/mL</th>
<th>CEA ≥6ng/mL</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>8</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>14</td>
<td>8</td>
<td>0.040</td>
</tr>
<tr>
<td>III</td>
<td>14</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>3</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>
Figure 8. Plasma levels of HCC-4 in CRC stages II-III with and without disease dissemination. The boxes represent median and quantiles, and bars minimum and maximum. Asterisks are extremes and circles are outliers.

Figure 9. Plasma levels of patients with CRC stage II, with and without disease dissemination.
Discussion

Paper I

In this study, the relationship between CRC patients with a family history of CRC in first-degree relatives and survival was analysed. We demonstrated that patients with colon cancer and positive family history had better overall- and cancer specific survival and prolonged time to recurrence compared to patients with negative family history. Several studies have shown survival advantage for patients with positive family history of CRC (145-150), while others concluded that positive family history has no impact or adverse effect on survival (151-153). There are indeed controversial statements as these studies are based on different designs, inclusion criteria, patient selection and heredity pattern. Two of the studies revealed the same results as ours in that colon cancer patients with positive family history of CRC, and not rectal cancer patients with positive family history, had improved survival (145, 147). Zell et al. had similar inclusion criteria for relatives as in this study, but their study was register based and the median age of the cohort was lower (147). The basis of survival benefits associated with familial CRC is though unclear. However in recent years increased attention has gained towards tumour biology. MSI tumours are associated with hereditary CRC, right-sided colon cancer and improved survival, and one explaining factor in the discrepancy of why colon cancer patients have improved survival might be due to MSI (122). We could also observe that a positive family history of CRC was associated with less advance disease stage. One explanation might be the high awareness of the disease in patients with positive family history of CRC and therefore detected in an early stage. However, none of the patients with positive family history of CRC was diagnosed during screening program and the multivariate analysis including disease stage did not affect the survival rate in patients with positive family history.

In clinical practice, the documentation of family history in patients with CRC is unfortunately incomplete, as was observed in this study and by others (154). It is crucial that patients with positive family history of CRC are identified, as there is a risk of missing families with hereditary CRC, and the benefit in survival when detected at an early stage.

As CRC is a common disease in the general population, the proportion of patients with positive family history of CRC has been reported between 16-19% (146-148, 151, 153). In the present cohort, only 10% of patients with
first-degree relative with CRC were identified. The proportion of patients with positive family history was low which is due to only including patients with verified histopathological reports of their relatives with CRC. In contrast to previous studies, the median age of patients with positive family history in this cohort was 75 years; and therefore, it was considered population based.

Patient-reported family history of CRC is accurate and reliable, but the sensitivity is lower (155, 156), as noted in the present study when one relative was discovered being operated for a benign adenoma instead of CRC. The strengths of this study were that the CRC diagnosis, in relation to first-degree CRC patients, was histopathological verified and that the cohort is prospectively sampled. The limitations were the small size of CRC patients with positive family history and that MSI status was unavailable.

**Paper II**

TrpRS was identified as a prognostic protein marker in CRC by HPA and further evaluated in a test cohort of 122 patients with CRC. A low intensity of TrpRS was correlated with worse outcome, which led to analysis of the present cohort. An increased expression of TrpRS was associated with improved prognosis and patients with low expression of TrpRS had more advanced tumour stage and increased risk of lymph node metastasis. The search for prognostic and predictive tumour markers in CRC is desirable. However, to date, CEA is still the only tumour marker recommended in clinical practice and is recommended for determining prognosis, surveillance after curative resection, and monitoring therapy in advanced CRC (91). In the present cohort, serum levels of CEA of ≥6 ng/ml were independently associated with an increased risk of recurrence and a trend for worse DFS in curatively treated CRC patients.

As an inhibitor of angiogenesis in both in vitro and in vivo studies (157-159), the role of TrpRS in the antiangiogenic pathway makes this protein an attractive choice for further studies in cancer therapy. The IHC expression of TrpRS in CRC has not yet been fully described, which means the results cannot be compared with other studies. As noted in the present study and by HPA screening cohort, the expression of TrpRS appeared to be lower in normal mucosa of the colon and rectum than for invasive primary tumour and lymph node metastases. This might reflect an increased angiogenic activity in tumour tissue. Low expression of TrpRS in primary tumours correlated with more advanced tumour and lymph node stages; this could be explained by increased neovascularisation, which in turn facilitate invasion of tumour cells thorough the bowel wall and improved ability of tumour cells to be implanted in the lymph nodes. Patients in stage III CRC with increased
expression of TrpRS in their lymph nodes had fewer recurrences than those with low TrpRS expression, although not statistically significant. The TMA technique is an efficient method for analysing multiple tumour tissues in a high-throughput fashion and in CRC, several molecular markers have been studied with this technique (160). However, there are some concerns with TMA studies as the TMA results are often difficult to reproduce due to variability in IHC staining and scoring methods (160). Therefore, it is important to confirm findings in TMA-based studies with other independent cohorts or methods. There are also some limitations related with IHC method, such as the validation of the antibody. The TrpRS antibody used in this study was previously validated by protein epitope signature tag array analysis with high specificity and a single band corresponded the predicted size (53 kDA) on the western blot. The potential prognostic value of TrpRS needs to be further evaluated.

Paper III

As colon cancer patients with positive family history of CRC had better survival (Paper I), the improvement in survival was evaluated to determine if it could be explained by MSI (Paper III). In this study, with longer follow-up, a better survival among patients with positive family history of CRC was confirmed, although the favourable prognosis for patients with positive family history of CRC could not be explained by MSI. Approximately 15% of the tumours exhibited MSI, which was in agreement with previous studies (161, 162). Patients with proximal colon cancer exhibiting MSI had prolonged CSS and TTR. Characteristics for MSI tumours were right colon localisation, female gender, mucinous histology, larger tumour size, age above 75 years, and less advanced disease stage: these characteristics were in agreement with finding from previous studies (163, 164). MSI tumours have increased tumour-infiltrating lymphocytes than MSS tumours, which may explain the improvement of survival among MSI patients (165). However, information about inflammatory response in our study was lacking, but would be of interest in future studies. Lanza et al showed that patients with MSI in disease stages II and III had better clinical outcome and that the survival advantage was more evident in disease stage III treated with surgery alone than in patients who received adjuvant chemotherapy (166). They also showed that patients in stage II MSI had a 97% of a 6-years survival rate, and, therefore they may not require adjuvant chemotherapy postoperatively (166). The favourable outcome in MSI tumours might be due to reduced likelihood of metastasis (167). MSI analysis was performed, previously as part of another study, in 32 randomly selected tumours of patients in stages II-III CRC. The proportion of
MSI-H tumours detected by MSI analysis was in accordance with IHC of MMR proteins. Although only few cases were investigated, to make a definite statement, IHC is a widely accepted and cost-effective method for detecting MSI in clinical practice (65, 168) with a sensitivity of 81-97% and specificity of 100%. A comparison of the sensitivity of IHC on TMA and full-face sections for MMR proteins of colon tumours from suspected carriers of defective MMR found that TMA-based analysis can be used as a prescreening method in suspected MMR defective carriers (169). There are technical limitations with TMA, such as loss of tissue cores during sectioning and staining procedures, and during IHC, such as increased background staining and nonspecific staining with the potential of false positive staining (170, 171). In this study, at first, 19 cases initially expressed uncertain staining pattern or tissue cores were not representative. These cases were reanalysed, resulting in only five cases being excluded from further analysis. The next preferable assignment would be further evaluation with MSI analysis of the MSI status in these five cases.

Tumours with KRAS mutations are associated with CIN pathway (129), whereas, BRAF mutations are more frequently occurred in MSI tumours (163). In our study, KRAS mutations were more common in patients with MSS tumours than in tumours displaying MSI, although this was not statistically significant. Furthermore, no cases of recurrences were identified in eight of the patients with distal colon cancer stages II-III exhibiting MSI. Although, rectal cancers displaying MSI are rare, they are often seen in hereditary CRC syndromes (83). In this study, no cases of MSI were seen in patients with rectal cancer. These data demonstrate the heterogeneous characteristics in CRC tumours.

**Paper IV**

The potential use of SP-PLA in the search for new prognostic biomarkers was demonstrated. The technique provides only a few microliters for protein analysis, as in this case 35 different antibodies. It enables efficient use of precious biobanked serum or plasma in search for new prognostic or predictive biomarkers in CRC. This study was a pilot study and the first to test SP-PLA on CRC patients. A strategic selection of cases was done to maximise the chances to obtaining potentially clinically valuable information from a limited number of samples. However, a small sample size give an insufficient power, which makes it difficult to draw any definitive conclusions about the plasma level of selected biomarkers and disease dissemination. The majority of selected biomarkers were detectable with SP-PLA and several of the selected biomarkers in the multiplex panel, are previously described to have important role in tumour biology and are of prognostic value in CRC (172, 173). Although a significant relationship between plasma lev-
els of selected biomarkers and disease stage could not be identified, low plasma levels of HCC-4 were present in patients with disease dissemination stage II-III. The activity of HCC-4 has shown to impair tumour cell growth in mouse adenocarcinoma cell line (174). Low plasma levels of TIMP-1 were present in stage II disseminated disease, and the patients were of younger age than patients with non-disseminated disease were. It has been mentioned that TIMP-1 plasma levels increases with age (175). These two statistically significant findings could reflect mass significance as many comparisons were done.

In this study CEA taken preoperatively was the marker of the highest significance; increased CEA levels were associated with advanced disease stage. This study was one of the first to test SP-PLA on plasma from patients with CRC; therefore, the interpretation of the results must be taken with caution. However, the same preselected biomarkers, as in this study, have been analysed with SP-PLA by Darmanis et al (144) on patients with cardiovascular disease and matched controls, of which three diagnostic markers for cardiovascular disease were identified. They concluded that SP-PLA can provide a platform for validation of diagnostic biomarkers in both biobanked samples and in clinical use (144). The method is rapidly developing and an analysis of 96 antibodies simultaneously is now available. However, the analysis of analysing many antibodies at the same time, such as multiple variables, tested increases the probability of some of the markers being increased or decreased in some of the groups analysed. SP-PLA could provide valuable guidance in the search for new prognostic and predictive tumour markers.
Conclusions

In this thesis, one population-based cohort was investigated to analyse the prognosis for patients with CRC in relation to heredity and tumour markers.

Paper I  Family history of CRC in first-degree relative in patients with CRC was an individual prognostic factor in patients with colon cancer. This improvement in survival could not be explained by known clinico-pathological factors.

Paper II  Patients with CRC and low expression of TrpRS had an increased risk of recurrence and poorer survival.

Paper III  Patients with proximal colon cancer and MSI had improved cancer specific survival. No correlation between MSI and heredity was found.

Paper IV  SP-PLA was a suitable method for protein detection and might provide valuable guidance in pursuing new prognostic and predictive biomarkers.
Future perspectives

The main aspects in this thesis were increased knowledge about heredity and identification of relevant tumour markers in relation to prognosis.

The relative risk of developing CRC increases if first-degree relatives are affected, and the risk further increases if there are known genetic mutations within the family. Earlier onset of the disease is more common among those with known heredity syndromes. Therefore, a regular follow-up regime in patients with known heredity syndromes is significant for early detection, which has impact on the outcome. However, documentation of family history of CRC in clinical practice is unfortunately incomplete, and there is the risk of missing families with hereditary CRC. An accurate taken family history (the size of the family, number of first-degree relatives with cancer, type of cancer and age at diagnosis) and risk assessment, in the clinical practice, forms the basis for eventual genetic testing. Collection of family data is time-consuming and sometimes inaccurate; especially where there is a lack of knowledge about the family history or the patients are of foreign origin, which makes it difficult to confirm family data. The identification of patients with familial CRC syndromes through only clinical criteria has low sensitivity, but this can be increased with additional genetic testing. However, genetic testing is also time-consuming, and can take up to 5-6 months before the results are known. Therefore, IHC is an alternative strategy, and is already used to identify patients with suspected Lynch syndrome (with the revised Bethesda guidelines). In some cases, normal IHC occurs despite a non-functional MMR protein. However, there are indeed challenges to overcome, such as the initial management of the patient in clinical setting, standardised screening program and follow-up regime, and simplification of the test procedure.

It is important to increase our knowledge of the molecular changes leading to development, recurrence, and metastasis of CRC through identifying potentially prognostic and predictive biomarkers. As CRC is a heterogenic disease, there is a need for better understanding in the role of genetic and epigenetic changes in a clinical setting in order to develop an optimal personalised cancer therapy, this is especially important in the selection of patients sensitive to chemotherapy in stages II and III CRC. In current practice, clinical and pathological factors can help to identifying high-risk patients,
although this does not always provide sufficient information to assess the recurrence risk for individual patients, or determine why some patients respond differently to the treatment. Molecular biomarkers have generated an increased interest in the choice of treatment. However, few biomarkers are clinically useful, although the validation of KRAS mutation status in metastatic CRC is an important finding. In addition, the prognostic and predictive value of MSI tumours, especially in stage II, needs to be clarifying, whether high-risk patients in stage II displaying MSI, require adjuvant chemotherapy. For better understanding of these issues, randomised clinical trails are therefore needed.

The introduction of high-throughput technologies has made it possible to assess the entire genome. Tissue microarrays enables high throughput analysis of protein expression in various tissues. The technique provides efficient analyses of potential biomarkers. However, there are several technical limitations in the construction procedure. The main problem is, particularly due to loss of tissue cores, which leads to missing data, and loss of statistical power, and may affect the ability to draw any conclusions. Another limitation is the number of cores needed for the representation of the tumour tissue with regard to tumour heterogeneity. Although two cores are considered sufficient for analysis of biomarkers, validation and standardisation of the technique is important. In the search for clinically useful biomarkers, various methods have been used for protein detection, with limited success. These methods are time-consuming, and expensive, require large amount of precious samples, and do not have adequate sensitivity and sensibility. New techniques, such as SP-PLA, are emerging; the advantages with this method are increased specificity, minimal sample consumption, and the capacity to analyse numerous targets in a multiplex format. In clinical use, SP-PLA can provide valuable guidance in the search for new prognostic and predictive biomarkers; however, the analysis of multiple biomarkers present a statistical challenge, to face when analysing multiple biomarkers, as it requires a large set of samples to achieve an adequate power.

The molecular classification of CRC also presents a challenge. The lack of available samples, the large patient numbers needed for an adequate power, lack of clinical significance, validated and standardised assays are tasks for research challenges that need to be overcome. Nevertheless, increased knowledge in the field of tumour biology and the on-going search for predictive and prognostic biomarkers yields valuable advantage in optimising a personalised treatment strategy for the CRC patient.
Bakgrund


Familjär kolorektal cancer

Livstidsrisken att utveckla CRC är ca 5% och denna risk ökar med åldern. Flertalet av alla CRC fall är sporadiska men upp till 20-25% har en familjär genetisk. Livstidsrisken förstärkas hos de med familjehistoria för CRC bland förstgradssläktingar (föräldrar/syskon/barn) och denna risk ökar ytterligare om insjuknandet sker under 45 års ålder. De två mest kända ärftliga synron men vid CRC är Lynch syndrom, även kallad hereditary non-polyposis colorectal cancer (HNPCC) och Familial adenomatous polyposis (FAP), båda med autosomalt dominant nedärvning. Lynch syndrom utgör ca 1-3% av all CRC fall och uppstå till följd av mutationer i DNA reparations (mismatch repair) proteiner (MLH1, MSH2, MSH6 och PMS2). Identifiering av dessa
bärare är viktigt då tumörer uppstår redan i 40-års ålder och det är även vanligt med tumörer i andra organ såsom livmoderslemhinna, äggstockar, mag- säcken, tunntarmar och urinvägar. Lynch syndrom tumörer är oftast lokalisera
till höger tjocktarm och är associerade med bättre prognos. Syndromet karakteriseras kliniskt enligt Amsterdam II/Bethesda kriterier, varefter diagnosen säkerställs med MSI-analys. FAP orsakas av mutation i adenomatous polyposis coli (APC) genen, och utgör ca 1% av all CRC fall. Syndromet kännetecknas av ett stort antal polyper i tjocktarmen och ändtarmen som utan behandling leder till cancerutveckling. Individer med FAP löper 100% risk att utveckla CRC. Polyper förekommer även i övre magtarmkanalen med ökad risk för utveckling av cancer i tolvfingerarmen. Genetisk mutationsanalys görs för att ställa diagnosen. Det är viktigt med systematisk upp- följning och när antalet polyper stiger rekommenderas profylaktiskt kirurgi för att förhindra utveckling av CRC.

**Molekylära mekanismer**

CRC uppstår genom ackumulering av mutationer eller inaktiviering av olika gener som leder till omvandlingen av normal cell till cancer cell, och denna process kan ta många år. Molekylärbiologiskt indelas CRC i flera grupper men de tre vanligaste är chromosomal instability pathway (CIN), microsatellite instability pathway (MSI) och CpG methylator phenotype pathway (CIMP). Majoriteten av alla sporadiska CRC fall utgörs av CIN-tumörer och karakteristiskt för dessa tumörer är ackumulering av mutationer i tumörsuppressor- och onkogener. MSI förekommer i ca 15% av fallen och uppkommer pga. inaktiverig av DNA reparationsproteiner. CIMP-tumörer uppstår pga inaktivering av tumörsuppressor- och DNA reparationsgener, och då framförallt i MLH1 genen. CIMP-tumörer uppvisar hög grad av DNA methyle- ring.

Samtliga nämnda tumörer har olika kliniska och molekylära egenskaper som kan ha betydelse i val av behandling och prognos.

Delarbete I


Delarbete II

Tryptophanyl-tRNA synthetase (TrpRS) är ett protein som är involverat i proteinsyntes, reglering av RNA transkription och translation samt inhibering av kärlnybildning. Proteinet uttrycks huvudsakligen i cellens cytoplasma i både cancerceller och normala celler. TrpRS har av human protein atlas (HPA) identifierats som en potentiell prognostisk markör varför antikroppen validerades på Västeråskohorten. HPA är ett storskaligt projekt som avser producera antikroppar mot samtliga humana proteiner (44 olika normalvävnad, 20 olika cancertyper och 46 olika cellinjer). Med hjälp av immunohistokemisk färgning på tissue microarray utvärderades proteintrycket hos patienter med CRC.

Låg TrpRS uttryck i tumörvävnad var korrelerad med ökad risk för lymfkörtelmetastas och avancerad tumörstadium. Patienter med ökat uttryck av TrpRS i tumörvävnad hade bättre överlevnad jämfört med patienter som hade lågt TrpRS uttryck. I en multivariat analys hade patienter med ökad TrpRS uttryck bättre sjukdomsfri överlevnad. Hos patienter med tjocktarmscancer och ökad TrpRS proteinuttryck sågs en minskad risk för sjukdomsåterfall. Sammanfattningsvis visade denna studie att lågt uttryck av
TrpRS är associerad med ökad risk för återfall och sämre överlevnad hos patienter med CRC. Dess anti-kärlnybildningsegenskap kan möjligen utnyttjas i utveckling av framtida potentiella läkemedel vid behandling av CRC.

Delarbete III

Microsatellite instabilitet (MSI) uppstår till följd av defekt DNA reparationsproteiner (MMR). MSI uppkommer i ca 10-20% av fallen vid sporadisk CRC. Då vi i delarbete I noterade att patienter med tjocktarmscancer och familjehistoria för CRC bland förstogradssläktingar hade förbättrat överlevnad, ville vi studera om den förbättrade överlevnaden kunde förklaras av MSI. Med hjälp av immunhistokemiskfärgning på TMA analyserades proteinuttrycket av MLH1, MSH2 och MSH6 i tumörvävnad från 320 patienter. Femton procent uttryckte MSI i tumörvävnad. Det sågs inget samband mellan patienter som uttryckte MSI i tumörvävnad och familjehistoria. Patienter med högersidigt tjocktarmscancer hade förbättrat överlevnad i sin cancersjukdom och förlängd tid till återfall. Åtta patienter med vänstersidigt tjocktarmscancer stadium II-III uttryckte MSI, ingen av dem fick återfall i sin sjukdom. MSI uttryck var associerad med högersidigt tjocktarmscancer, kvinnligt kön, mucinös histologi, större tumörer och hög ålder. I tumörvävnad från ändtarmen fanns inga fall med MSI. Sammanfattningsvis visade denna studie att patienter som uttrycker MSI har förbättrad canceröverlevnad och längre tid till återfall. Den förbättrade överlevnaden hos patienter med familjehistoria för CRC kunde inte förklaras av MSI.

Delarbete IV

Solid-phase proximity ligation assay (SP-PLA) är en ny metod som används för detektering av många proteiner samtidigt, ur en liten mängd plasma. Syftet med denna pilotstudie var att utvärdera SP-PLA på 60 strategiskt utvalda patienter med (n=31) och utan (n=29) spridd sjukdom, stadium I-IV CRC. Trettiofem redan förvalda proteiner, involverade i cancer, inflammation och hjärt-kärlsjukdomar, analyserades ur 5 µl plasma med SP-PLA. Carcinoembryonalt antigen (CEA) tidigare taget på samma kohort, användes som referens. Tjugo av 35 proteiner detekterades med denna metod. Låg plasma koncentration av HCC-4 noterades hos patienter i stadium II-III med spridd sjukdom. Patienter med spridd sjukdom i stadium II hade låga plasma koncentrationer av TIMP-1. Höga CEA nivåer korrelerade med spridd sjukdom och var den markör som gav bäst prognostisk information. Detta är den första studien som har utvärderat SP-PLA metoden på CRC patienter, varför resultaten måste tolkas med försiktighet. Sammanfattningsvis visade denna studie att SP-PLA är en användbar metod för proteindetektering och kan vara värdefull i jakten på nya tumörmarkörer.
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