TISSUE REMODELLING PROTEASES AS PROGNOSTIC FACTORS IN COLON AND RECTAL CANCER

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ABSTRACT

Background: Colorectal cancer is the third most common cancer in Sweden and the main treatment is surgery. The TNM classification is the principal staging tool, although insufficient in identifying all patients with poor survival. The identification of molecular prognostic markers would be important in order to further aid in the identification of these patients. Components participating in the remodellation of extracellular matrix were analysed for their association with tumour progression and survival.

Methods: Patients with colorectal cancer were included in the studies during 1999-2004. Protein expression was evaluated by ELISA technique and immunohistochemistry, and related to tumour classifications. The association with cancer specific survival (CSS) was analysed by Cox proportional hazard analysis and differences between survival curves (Kaplan-Meier method) were evaluated by the Log Rank test.

Results: The expression of all measured markers were significantly higher in tumour tissue compared to tumour free mucosa. Matrix metalloproteinase-1 (MMP-1) protein expression in tumour tissue and MMP-2 expression in plasma was associated with increasing tumour stage (T-status) and lymph node metastasis in patients without distant metastatic disease. When survival data were analysed, MMP-2 in tumour tissue and MMP-1 and -9 expression in adjacent tumour free mucosa were associated with CSS in colon cancer. The association with CSS was maintained for MMP-1 in multivariate analysis also in patients without distant metastatic disease. High levels of urokinase Plasminogen Activator (uPA) expression in tumour free mucosa were associated with improved survival, but only in patients with rectal cancer. uPA expression below the chosen cut-off value identified M_0 patients with increased risk of poor survival. TGF-beta1 and PAI-1 protein expression was associated with metastatic disease and the survival analysis confirmed these results.

Discussion: Results indicate that the association of systemically measured factors with survival is due to their strong correlation with metastatic disease. These findings might reflect a generalised response to the metastatic disease. The differential association of MMP-1, MMP-9 and uPA expression with cancer specific survival in adjacent tumour free mucosa in colon and rectal cancer was unexpected. This also means that prognostic information could be available already in the preoperative setting, which could open up the opportunity to offer neo-adjuvant therapy to high-risk patients. The results suggest that the macroscopically normal mucosa in the tumour-bearing segment reflects local tumour progression, and it seems evident that important changes in the microenvironment, even remote from the tumour, are present.

Key words: extracellular matrix, survival, staging, colorectal cancer, mucosa, stroma

LIST OF PUBLICATIONS

I. Marcus Langenskiöld, Lena Holmdahl, Peter Falk, Marie-Louise Ivarsson. Increased MMP-2 protein expression in lymph node positive patients with colorectal cancer. Int J Colorectal Dis. 2005 May;20(3):245-52.

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TABLE OF CONTENTS

ABBREVIATIONS

AJCC	American joint committee on cancer
APC	Adenomatosis polyposis coli
BM	Basement membrane
CEA	Carcinoembryonic antigen
CIN	Chromosome instability
CME	Complete mesocolic excision
CRC	Colorectal cancer
CSS	Cancer specific survival
DCC	Deleted in colorectal cancer
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
FLV	Fluorouracil/Leucovorin
5-FU	5-Fluorouracil
HR	Hazard ratio
HRP	Horseradish peroxidase
Htx-Eo	Hematoxylin-Eosin
LNR	Lymph node ratio
LV	Leucovorin
MRI	Magnetic resonance imaging
MSI	Microsatellite instability
MSS	Microsatellite stable
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
PAI-1	Plasminogen Activating Inhibitor-1
PLSD	Protected least significant difference
PPS	Plasminogen/plasmin system
ROC	Receiver operating characteristics
TGF-β1	Transforming growth factor-β1
TIL	Tumour infiltrating lymphocyte
TIMP	Tissue inhibitor of matrix metalloproteinase
TMB	Tetrametylbenzidine
TME	Total mesorectal excision
TNM	Tumour / Node / Metastasis classification
t-PA	tissue-type Plasminogen Activator
UICC	International union against cancer (Union international contre le cancer)
uPA	urokinase Plasminogen Activator
uPAR	urokinase Plasminogen Activator receptor

INTRODUCTION

COLORECTAL CANCER

Epidemiology

Colorectal cancer is one of the most common malignant diseases in Sweden, accounting for 11% of all malignant cases per year. In 2004, 5670 new cases of colorectal cancer were registered (in a population of about 9 million). Colon cancer is more common and accounts for 60% of all colorectal cancer cases in males and 68% in females. The average age in Sweden at diagnosis is 72 years. The incidence of rectal cancer is approximately 20/100000 and in colon cancer 40/100000 [1]. Colorectal cancer is, despite improved surgery [2] and modern neoadjuvant and adjuvant treatment, still associated with a significant mortality rate [3]. In Sweden, the overall 5-year cancer specific survival (CSS) for patients with rectal cancer is reported to be 54% for women and 60% for men. Similar figures are reported for patients with colon cancer (57% and 59% respectively) [1].

Surgery in colorectal cancer

The main therapy in colorectal cancer is surgery, and adjuvant therapy is added depending on the pathological and anatomical diagnosis. In Sweden, rectal cancer patients have been offered neo-adjuvant radiotherapy totalling 25 Gy in five fractions [4]. Later reports have confirmed the beneficial effect of local radiotherapy regarding local recurrences but not for survival [5,6]. No standardised neo-adjuvant therapy is given to colon cancer patients.

The surgical approach in colon and rectal cancer differs. The operation for colon cancer has been the most common intra abdominal operation for malignant disease for general surgeons and is characterised by a segmental resection of the tumour and adjacent tumour free bowel, mesocolon, artery and vein. The resections performed are right-sided hemicolectomy, resection of the transverse colon, left-sided hemicolectomy and resection of the sigmoid colon. The procedure for rectal cancer is total mesorectal excision (TME), performed as an abdomino-perianal resection or anterior resection depending on the tumour position in relation to the anal verge, and is a procedure for specialised colorectal surgeons, which has improved results of rectal surgery [2,5]. TME is characterised by the preservation of the peri-rectal fascia and the removal of the complete mesorectum. The technique of TME is based on sharp dissection under direct vision following the fascial surface of the mesorectum. This plane is usually avascular and enables identification and preservation of the autonomic nerve plexus [7].

The TME technique has been widely accepted as state of the art, although the surgical approach in colon cancer surgery has not received the same attention compared with rectal cancer. However, the issue of the different anatomical approach in rectal cancer compared

to colon cancer surgery has been addressed in recent years. In an attempt to further improve results, Complete Mesocolic Excision (CME), has been proposed in colon cancer surgery, in line with the surgical principles implemented in rectal cancer surgery [8]. Considering these facts, results have improved through centralised surgery to specialised colorectal units. Modern surgical technique, preserving the fascias and peritoneal margin of the resection (mesorectum in rectal cancer and the segmental mesocolon in colon cancer), high-ligation of artery and vein are most likely key aspects in this development. And most importantly, a multidisciplinary team of surgeons, oncologists and pathologists that are participating in the pre- and postoperative treatment decisions.

Survival and Prognosis

Staging Systems

Preoperative evaluation of newly diagnosed colorectal cancer (CRC) primarily relies on radiological investigation and postoperative classification of anatomic distribution of disease that is based on the International Union Against Cancer (UICC-TNM) and American Joint Committee on Cancer (AJCC) classifications [9,10]. Tumour depth, lymph node involvement, generalized metastases and tumour differentiation are still the most important morphological prognostic factors.

Current recommendations suggest that Magnetic Resonance Imaging (MRI) should be used in assessing the depth of tumour invasion in rectal cancer in order to correctly evaluate the need for preoperative radiotherapy in patients with rectal cancer [1]. However, MRI was not standard of care in the preoperative evaluation during the period the patients in this thesis were included (1999-2004).

The anatomy of the large intestine in relation to tumour classification

The bowel wall is defined by following layers; the mucosal layer (epithelial cells and basement membrane, lamina propria and muscularis mucosa), the submucosa, the external

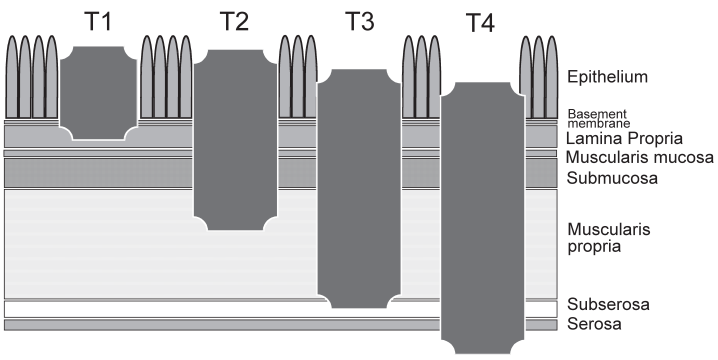

Fig. 1. Tumour depth is displayed as the TNM(T) classification. T1 tumours are limited to the submucosal layer, where as T4 tumours are characterized by tumour growth beyond the serosal layer.

muscle layer and the peritoneal (serosa) outer layer facing the abdominal cavity. These anatomical structures are the basis for the current staging systems in use (Fig. 1). As the depth of tumour invasion increases, the risk for nodal and distant metastasis increases.

The investigation of the resected specimen defines the 3 N categories: N0 (no lymph nodes involved), N1 (1–3 lymph nodes involved), and N2 (>3 lymph nodes involved). Current guidelines recommend the identification of 12 or more lymph nodes in the resected specimen [11], as the examination of fewer regional lymph nodes has been associated with stage migration and subsequent poorer outcome in patients both with node-negative and node-positive disease [12,13].

It is generally believed that the examination of fewer lymph nodes may reflect an insufficient surgical procedure or a qualitatively poor pathological examination of the specimen. This can lead to an incorrect under-staging, thereby excluding the patient from beneficial adjuvant treatment.

The TNM classification is the base for the UICC and AJCC classifications, which are often used in the clinical practice (Table 1).

Prognosis

Table 1. The UICC-classification in relation to the TNM-classification

UICC	TNM
I	$pT_{1-2}N_0M_0$
II	$pT_{1-4}N_0M_0$
III	$pT_{1-4}N_{1-2}M_0$
IV	$pT_{1-4}N_{0-2}M_1$

Despite improvements in surgical techniques, adjuvant and neo-adjuvant chemotherapy, the 5-year survival rate for patients with CRC ranges from 5-90% with tumour progression (stage I: 90-95%, II: 75-85%, III: 50-60% and IV: 0-10%). The prognosis for patients without distant metastatic disease varies from 50-95% depending on the tumour stage [14].

The correct staging of each patient is crucial in order to plan an optimal treatment regimen. It is widely recognised that prognostic information based on clinical and histopathological investigation is insufficient, although tumour stage and lymph node involvement are the main prognostic tools in evaluating cancer specific survival. It is questionable to expose a large number of patients to adjuvant treatment with considerable side effects without indications that they will benefit from such treatment. Finding molecular markers to better identify patients with higher risk for poor survival [15,16] would be valuable in order to customise pre- and postoperative treatment as well as enabling closer follow-up for these patients.

ADJUVANT TREATMENT

Background

The principal treatment for colorectal cancer is surgery. The role of chemotherapy is mainly in the adjuvant setting and has a modest effect in colorectal cancer. Fluorouracil (5-Fu) is the primary systemic treatment for colorectal cancer. It is a fluorinated pyrimidine which inhibits thymidylate synthetase, the rate-limiting enzyme in pyrimidine nucleotide synthesis [17]. 5-Fu is commonly combined with leucovorin (LV), which is believed to enhance the interaction of 5-Fu with this enzyme [18,19]. Pooled analysis of several randomized trials of postoperative fluorouracil-based therapy versus surgery alone have shown an increase in 5-year disease-free survival from 42% to 58% and a 5-year overall survival from 51% to 61% in patients with stage III disease [20]. It has also been shown that the addition of levamisole to fluorouracil and leucovorin does not improve survival [21,22]. Oral fluoropyrimidines have been evaluated in the adjuvant setting in colon cancer, and are effective in stage III colon cancer [23]. In a large randomised study (MOSAIC) in stage III colon cancer patients, treatment with either 5-Fu/Leucovorin (FLV) alone or with the complement of Oxaliplatin was compared. The study suggested a nearly 9% increase in disease free-survival with the addition of Oxaliplatin to the FLV regimen [24].

Irinotecan (Campto™) has shown to be of value in patients with metastatic disease [25,26]. However, in the adjuvant setting, irinotecan has shown increased side-effects without improved results [27]. Other treatment modalities under current investigation are angiogenesis inhibitors (bevacituzimab (Avastin™)) and epidermal growth factor receptor inhibitors (i.e. Cetuximab (Erbitux™)). However, the role of these modern chemotherapeutic agents in the adjuvant setting is unclear and currently under investigation [28].

The adjuvant treatment of rectal cancer has mainly been based on the studies made in colon cancer patients, and level I data has been scarce, although sufficient amount of data exist regarding neo-adjuvant radiotherapy and radiochemotherapy [29]. During the current study period (1999-2004), 5Fu/Leucovorin was standard of care, and only patients with distant metastatic disease in the current studies have received irinotecan or oxaliplatin as palliative treatment. Forty-eight percent of colon cancer patients and 56% of rectal cancer patients that were UICC stage III in our studies received adjuvant chemotherapy.

Use of adjuvant treatment for stage II colorectal cancer

For patients with stage II colon cancer, the use of adjuvant chemotherapy remains controversial, but may be appropriate in a subset of individuals at higher risk for disease recurrence. An increased risk is expected in T4 staged tumours, tumours with bowel

perforation or if the analysis of the number of investigated lymph nodes is incomplete in the resected specimen [12,30]. Data from a large Scandinavian study during 1991-1997 did not support adjuvant treatment of stage II colorectal cancers [31]. At this time, the national Swedish treatment program for colorectal cancer does not support the general use of adjuvant chemotherapy in stage II colorectal cancer [1].

PROGNOSTIC MARKERS

The evaluation of future risk for recurrent disease and subsequent poor survival of newly diagnosed colorectal cancer relies predominantly on staging that is defined by the UICC-TNM and AJCC classifications. However, the specificity of information based on clinical and histopathological investigations is insufficient to fully estimate the risk for recurrence and poor disease specific survival. Selected patients without lymph node metastasis are likely to benefit from adjuvant chemotherapy, but cannot be properly identified.
The UICC and AJCC classifications remain gold standard in predicting the outcome in colorectal cancer.
In recent years numerous studies have addressed the issue of the quality of the pathological report. The number of investigated lymph nodes is of importance in order to correctly define the TNM classification in each patient [12]. In line with these findings, also the lymph node ratio (the number of cancer positive lymph nodes/the number of identified lymph nodes; LNR) has also been shown to perhaps more accurately identify patients with risk of poor prognosis compared with the traditional N-stage, where only the presence and number of cancer positive lymph nodes are evaluated [13]. At present, LNR is only used in the research setting, although its use might increase in clinical practice in the future.

Molecular prognostic markers

Many serum biomarkers are associated with disseminated disease, and the association with disease specific survival is less pronounced in the subgroup of patients without distant metastasis at the time of surgery [32]. Finding molecular markers to identify high-risk patients early would be valuable to be able to optimise treatment, but how these patients are best identified is not well understood [16,33].

Carcinoembryonic Antigen

The utility of the Carcinoembryonic Antigen (CEA) as a prognostic factor has been under rigorous investigation during the last decades. The clinical value of CEA has been carefully evaluated, and results indicate that CEA is useful primarily in identifying patients with recurrent disease after curatively intended surgery during follow-up [9]. Results have also suggested a prognostic value of CEA in the preoperative setting [34-36].

Microsatellite Instability

It is believed that 75-80% of microsatellite stable (MSS) colorectal tumours arise from a pathway defined by aneuploidy, allelic losses, amplifications, translocations and mutation of APC, K-ras and P53. The prognosis of MSS colorectal cancer is dependent on TNM stage, although the prognosis for tumours belonging to the same UICC stage differs considerably. The remaining tumours are characterised by microsatellite instability (MSI), which is defined by inactivation of mismatch repair genes. The mutations of these genes are also associated with the loss of the Transforming growth factor-β1 (TGF-β1) receptor [37]. As TGF-β1 with increasing tumour load has an oncogenic effect, better survival in patients with MSI tumours compared with patients with MSS tumours is linked to the dysfunction of the TGF-β1 receptor [38]. However, these results are derived from retrospective studies, and the benefit of MSI status in clinical use is not defined.

LOH18q

The long arm of chromosome 18 contains important genes in colorectal cancer pathogenesis. Chromosomal loss at 18q has been associated with up to 70% of CRCs. The chromosomal loss is believed to be one of the corner stones of the adenoma-carcinoma sequence, although this model is increasingly debated [39]. The DCC gene, which maps to 18q21 and codes for a neutrin-1 receptor, is believed to be a key player in colorectal carcinogenesis due to its role in apoptosis. The chromosomal loss at 18q is associated with shorter survival [40]. However, chromosomal loss as a useful prognostic marker in evaluating the risk for poor survival has not proved valuable in clinical practice.

P53

The P53 tumour suppressor gene has been under investigation for many years. Approximately 50% of colorectal tumours have mutations in the P53 gene, which has been associated with poor survival. However, the prognostic value of P53 has not been clinically meaningful and it is not recommended for either prognostic use or disease surveillance [9].

Tumour immunity

Various immune/inflammatory cells, usually along the invasive margin, infiltrate human colorectal cancer tissues. However, results indicate that these cellular responses, particularly lymphocytic reactions, are independent prognostic factors for survival. Lymphocytes are recognised as small round cells by Htx-Eo stained sections. However, these cells are usually differentiated from plasma cells, neutrophils, eosinophils, macrophages or mast cells by their histological features. One of the pioneering works by Jass *et al* demonstrated, that infiltration by lymphocytes along the invasive border of rectal cancer is an independent prognostic factor for improved survival [41]. These results indicate that the microenvironment of the tumour is of fundamental importance in tumour invasion and progression. Although tumour infiltrating lymphocytes (TILs) have been especially associated with MSI tumours [42], the prognostic value of TILs seems to be

restricted to MSS tumours [43]. This suggests a difference in the biological environment for rectal cancers compared to colon cancers, as MSI tumours primarily are seen in the proximal colon. Although interesting, the use of TILs is not recommended in clinical practice.

THE ROLE OF EXTRACELLULAR MATRIX IN TUMOUR BIOLOGY

To enable tumour progression and metastasis, the tumour has to invade anatomical tissue borders, such as basal membranes and the interstitial stroma (Fig. 2). For tumour invasion to occur, extracellular matrix has to be degraded, and proteolytic enzymes mediate this process. Extracellular matrix-degrading proteolytic enzymes can be divided into four subgroups according to their amino acid residue or cofactor required for catalytic activity: cysteine proteases, aspartic proteases, serine proteases and metalloproteinases, which contain a metal ion in the catalytic site [44]. The serine proteases and metalloproteinases are discussed in this thesis.

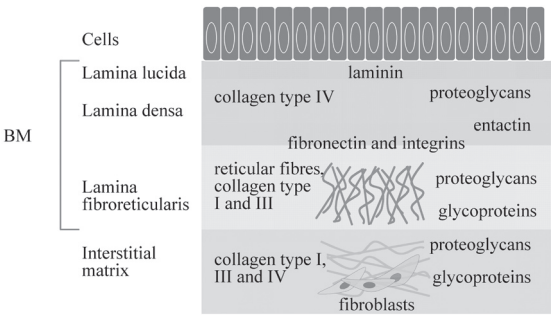

Fig. 2.
The Extracellular matrix (ECM) is composed of the interstitial matrix and the basement membrane (BM).

Proteases are produced in different cell types, such as tumour cells, fibroblasts, tumour-associated monocytes and polymorphonuclear lymphocytes [45,46]. Cancer cells usually can modify their environment by producing stroma-modulating growth factors. The interplay between the tumour and the desmoplastic stroma is believed to play an important role in enabling tumour invasion and metastasis. Growth factors associated with extracellular stroma remodellation are fibroblast stimulating growth factor (FGF), members of vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), epidermal growth factor receptor ligands (EGFR), interleukins and transforming growth factor-β1 (TGF-β1). These factors also activate surrounding stromal cell types, such as fibroblasts and smooth-muscle cells [47].

Collagen are sub typed into several groups, type I, II, III and IV being the most common. Collagen type IV is the main component of the basement membrane (BM) [48], which is important in tumour invasion [49]. The BM consists of the outer layers; lamina fibroreticularis and lamina lucida and the inner layer (lamina densa). The structure of the BM has been debated, but electron microscopic investigation techniques have been useful in describing the three layers composing the BM [50].
Laminin, fibronectin, proteoglycans and glycosaminoglycans are other major glycoproteins in the extracellular matrix [51]. The functional overlap of the MMPs is significant.

Therefore, it is widely accepted that the complete range of MMPs can degrade all ECM components [48]. The MMPs are secreted as inactive pro-enzymes and activation is needed in order to achieve proteolysis [45].
In summary, degradation of extracellular matrix is needed for cell migration and tumour invasion. Serine proteases and their inhibitors and especially MMPs are believed to be an essential part of this dynamic process.

Regulatory components of the extracellular matrix

Plasminogen/Plasmin System

Fibrinolysis depends on the balance between the members in the plasmin/plasminogen system (PPS). In order to initiate fibrin degradation, inactive plasminogen has to be activated to plasmin. There are two different types of plasminogen activators, tissue-type (t-PA) and urokinase (uPA) plasminogen activator, that catalyses the conversion of the inactive precursor plasminogen to the active proteinase plasmin. Plasmin can degrade most extracellular proteins through activation of matrix metalloproteinases (MMPs). In the systemic circulation this is achieved by t-PA while the uPA system controls degradation of extracellular matrix primarily in tissue, which is important for tissue remodelling.
uPA is a 52 kD serine proteinase that binds to a specific cell surface receptor (uPAR). The importance of uPA in relation to tumour growth has been shown in several different cancers and is reported to be associated with poor prognosis [52-56].
Plasminogen activator inhibitor-1 (PAI-1) is a member of the serine proteinase inhibitor (SERPIN) family and is the primary physiological inhibitor of both t-PA and uPA [57]. It circulates as a complex with the adhesive glycoprotein vitronectin [58]. The binding of uPA to uPAR stimulates intracellular signalling and is associated with cell adhesion [59]. PAI-1 is a 45 kDa serine proteinase, which acts as a fibrinolytic inhibitor [60]. It has been associated with tumour dissemination and poor prognosis in several tumour forms [61-64], opposed to its originally assumed role [65-67]. Additional mechanisms by which PAI-1 can regulate tumour growth is by stimulating cell migration and apoptosis [68,69]. Paradoxically, a high level of PAI-1 in tumours has been shown to be an unfavourable prognostic factor [70,71].

Matrix metalloproteinases

The matrix metalloproteinases (MMPs) are a group of Zn^{2+} or Ca^{2+} dependent proteases, whose function is to degrade components of the extracellular matrix. At least 28 MMPs are identified, of which 6 are transmembranous. The fibrinolytic system is, by the activation of MMPs through active plasmin, key players in the progression of different malignancies [44,72-77]. MMPs belong to a large family of proteinases, which can degrade different components of extracellular matrix, e.g. collagens, proteoglycans, laminins and fibronectins. These in turn are inhibited by three different “tissue inhibitors of metalloproteinases” (TIMPs). The MMPs are involved in extra cellular stromal breakdown

in both pathological and normal situations. Previously MMPs were thought to be important in invasion and metastasis mainly by the degradation of the basement membrane and the ECM. It is evident that the role of MMPs is not restricted to degradation, but MMPs have also important roles in the activation of a number of cytokines, including growth factor precursors and receptors, tyrosine kinase receptors, cell adhesion molecules and other proteases that modify tumour environment [45].

Transforming growth factor-β1

Transforming growth factor-β1 (TGF-β1) is a dimeric polypeptide belonging to a large family of related proteins [78-83]. TGF-β1 can be activated by cell-bound matrix metalloproteinases, and regulates tumour invasion and angiogenesis [84,85]. TGF-β1 controls proliferation and differentiation in many cell types [86]. TGF-β1 has also a role in preserving epithelial tissue organisation, preventing early transition from organised hyperplasia to dysplasia and thereby inhibiting early stage tumorigenesis [87]. However, TGF-β1 appears to have the opposite function in more advanced tumour stages, as changes in TGF-β1 expression and signalling seems to promote tumour progression. These findings are usually explained as the dualistic character of TGF-β1, where the suppressor functions of TGF-β1 are found in early tumorigenesis and the oncogenic effects are seen in a later (metastatic) phase of tumour progression [37]. The suppressor capabilities are derived, in part, from the ability of TGF-β1 to inhibit cell growth in normal cells [88].

AIMS OF THESIS

The aims of the thesis were to:

- Evaluate whether the expression of molecular markers capable of degrading extracellular matrix covariates with known tumour staging classifications.

- Evaluate the prognostic associations measured as cancer specific survival of these markers in colorectal cancer.

- Evaluate whether the prognostic association of these markers differs between colon and rectal cancer.

- Evaluate whether any of these markers can identify individuals with high risk of disease specific death after curative surgery.

MATERIAL AND METHODS

PATIENTS

Study patients

Patients with colorectal cancer were prospectively included between 1999-2004. Patients who received neo-adjuvant radiotherapy due to rectal carcinoma where excluded as irradiation causes a local reaction in the tissue marked by inflammation and thus is a confounding factor when assessing the expression of tissue remodelling proteases [89,90]. The total number of patients included was 221 (colon cancer n=156, non-irradiated rectal cancer n=65). None of the participating patients in the studies had neo-adjuvant chemotherapy in accordance with the institutional treatment protocol. All patients received one standardised dose of prophylactic antibiotics and prophylaxis against thrombosis with low-molecular-weight heparin. Informed consent was obtained from all included patients, and the studies were approved by the local Ethics Committee.

Non-study patients during the time period

Rectal cancer

The exclusion of subjects who received radiotherapy could have introduced selection bias. An analysis was therefore undertaken to assess the magnitude of this potential effect. One effect is that there are more patients with disseminated rectal cancer in the study population, as they are not eligible for pre-operative radiotherapy. Furthermore, based on eligibility criteria for radiotherapy one would expect that there was a divergent distribution of T-stage between the irradiated and the non-irradiated population. Patients that received preoperative radiotherapy were included in other studies during the same study period and were compared with patients included in this thesis. Much to our surprise, the two populations were comparable in terms of T-stage distribution (unpublished data). It is therefore reasonable to assume that the results obtained in the population we investigated could be generalisable. However, results also suggest that the pre-operative selection of patients for preoperative radiotherapy during the study period might have been inadequate.

During the study period, a total of 344 subjects with rectal cancer were not included in the studies, and 223 of these subjects did not undergo radiotherapy. To investigate how this could influence conclusions, we compared characteristics of this population with the study population. The information that was available on the non-study patients was UICC stage. No significant differences were observed in UICC distribution between patients included in the studies compared to patients outside the studies at our centre during the study period.

This means that about 2/3 of all patients did not take part of the study. No significant differences were observed in UICC distribution between patients included in the studies compared to non-study patients during the study period.

Colon cancer

Information on non-study patients was not available in patients with colon cancer. Instead, demographic data from all patients surgically treated at the Department of Surgery, Sahlgrenska University Hospital/Ostra during the study period were analysed. A total of 649 patients with colon cancer had their tumour surgically resected. Therefore, about 2/3 of all patients did not take part of the study. No significant differences were observed in UICC distribution between patients included in the studies compared to all patients treated at the surgical department during the study period.

Paper I

Seventy-two patients who underwent surgery for a colorectal carcinoma were included in the study between February 1999 and September 2000. The average age of the patients with rectal cancer was 74 years and 72 years for patients with colon cancer. The average age of all patients was 73 years.

Paper II and IV

A cohort of 169 patients who underwent surgery for a colorectal carcinoma between February 1999 and June 2003 were prospectively included in the study. The mean age of patients with colon cancer was 73 years (range 42-91) years and for rectal cancer patients 75 years (range 51-89).

Paper III

A cohort of 221 patients who underwent surgery for colon and rectal cancer during the period February 1999 to March 2004 was prospectively included.
The mean age of colon cancer patients was 72 years (range 31-93) and 75 years (range 38-89) for patients with rectal cancer. Eighty-one (52%) of the patients with colon cancer (n=156) and 37 (56%) of rectal cancer patients (n=65) were males.

TISSUE SAMPLING & PROCESSING

Optimising blood and tissue sampling: the pilot study

Before commencing the study we performed a pilot study including 5 patients with colorectal cancer. The results demonstrated that biopsies had to be processed immediately for mRNA assessment. mRNA degraded if the biopsy was obtained on average 40 minutes after resection, compared to immediately after resection. However, protein quality seemed unaffected by the delay (unpublished data). Strict biopsy retrieval and placement of the biopsies in liquid nitrogen was therefore employed in the studies. A sample setup was prepared and in place in the operating theatre through all the studies in order to minimise the risk for tissue degradation.

Blood samples

Blood samples were taken in a standardised way after induction of anaesthesia.
In order to minimise platelet-associated contamination, citrate tubes were utilised for sample collection, as the use of EDTA tubes can be associated with increased platelet associated contamination [91-93]. Venous blood was collected in sodium citrate and Diatube (CTAD), (BD, Franklin Lakes, NJ, USA) tubes, then centrifuged and the supernatant frozen at –80°C until assayed.

Tissue samples

Surgical biopsies were taken immediately after resection in the operating theatre. Each biopsy measured approximately 1 cm^2. Three biopsies were taken from each patient. One biopsy was taken from the macroscopically tumour-free bowel segment, approximately 10 cm from the tumour, and a second biopsy from the tumour itself. The necrotic tumour centre was avoided during the biopsy procedure. The tissue samples were snap frozen in liquid nitrogen in the operating theatre. A third biopsy was taken from the macroscopical tumour borderzone for immunohistochemical assay and put in Bouin's solution.

TISSUE AND BLOOD PROCESSING

After thawing, samples were weighed and homogenised using an Ultra-Turrax (24 000 rpm) in PBS buffer containing 0.01% Triton X-100 using 1 ml buffer per 40 mg of tissue. The homogenate was centrifuged (10 000 g, 3 minutes) and the supernatant collected and frozen at –80°C until assayed for protein as previously described [94].
The number of operators was kept to a minimum to standardise the procedures. Two operators did all homogenisations and protein extractions.

Protein analysis

Enzyme-linked immunosorbent assay (Paper I-IV)

Commercially available enzyme-linked immunosorbent assay (ELISA) was used to detect and quantify analysed proteins. The antigen is attached to pre-coated plates and excess antigen is washed. A secondary antibody specific for the antigen together with a linked enzyme is then attached to the plate. By using an antibody-antigen reaction, as well as an enzyme reaction, this technique converts a peroxidase sensitive substrate into a colour. The absorption at a specific wavelength was quantified by spectrophotometer (V-max, Molecular Devices) to measure concentration. Internal standards of known concentrations are used to quantify the optical densities of the samples. The principal mode of action of the ELISA is shown in (Fig. 3).

Variability of the assays

Experienced laboratory technicians performed the assays and all samples were analysed in duplicates. To minimise inter-assay variability, two operators performed the assays. Quality control of the assays included samples with known concentrations and a reagent blank on each plate. Further, each plate had several control samples with known concentrations, that were used in several ELISA plates in order to evaluate inter-assay variability. To standardise the results, the concentrations of measured markers were normalised to the total protein content of each sample [95]. Assays of total protein content were performed using a chromogenic assay (DC Protein assay, Bio-Rad Hercules, CA, USA).

Intra- and inter-assay variations

Intra-assay variations are variations between samples within the same ELISA plate and inter-assay variations are variations between the analysed plates. These variations are defined as the product of the standard deviation divided by the mean (the coefficient of variation) expressed as %. According to the manufacturer, the intra-assay variations for uPA and PAI-1 were below 9%, for MMPs between 3-7%, and for TGF-β1 4%. Similarly, the inter-assay variations for uPA and PAI-1 were 5-8%, MMPs 6-9% and 11.6% for TGF-β1. In our laboratory similar or lower intra- and inter-assay variations were observed.

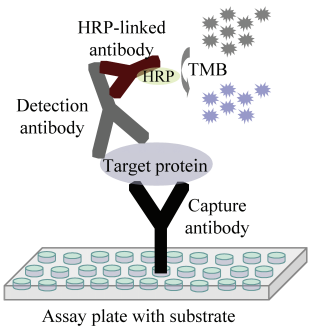

Fig. 3.
The mode of action for the enzyme linked immunosorbent assay (ELISA) is characterised by the use of several specific antibodies to measure protein concentration. After the application of the horseradish peroxidase (HRP) linked antibody, substrate (tetrametylbenzidine, (TMB)) is added and the absorption is quantified by spectrophotometer. Illustration in co-operation with MD E Angenete.

Immunohistochemistry (Paper IV)

Biopsies taken from the borderline of the tumour and the adjacent tumour free mucosa during surgery were fixed overnight in Bouin's solution (Sigma Diagnostic, St Louis, MO, USA). Following wash with phosphate buffered saline solution biopsies were dehydrated in increasing ethanol gradients and xylene prior to paraffin fixation. Sections (4-6 µm) were deparaffinised and stained with Haematoxylin & Eosin for morphologic assessment. For immunological evaluation antibodies towards MMP-1 were examined. Primary mouse antibodies against human MMP-1 diluted to 1 µg/mL (#IM35L, 1:100, Calbiochem, Oncogene Res Products, Cambridge, MA, USA) were used together with the DAKO Envision system (DAKO Cytomation, Glostrup, Denmark). The signal was detected with a chromogenic substrate (diaminobenzidine) according to the manufacturers instructions. A negative control consisted of incubations of tissue sections with mouse IgG directed towards an enzyme that is neither present nor inducible in mammalian tissues (X-0931, DAKO Cytomation, Glostrup Denmark). Counterstaining with Haematoxylin-Eosin was used prior to dehydration and mounting with cover slips. Evaluation of distribution and qualitative comparison was performed using Nikon Eclipse 50i microscope together with Nicon Eclipse E1000M and Kontron Elektronik/Prog/Res/3012 digital photo equipment.

STATISTICS

Paper I

Due to the limited number of patients in the different stages, non-parametric tests were used, as a non-normal distribution was assumed. Friedmans test was used regarding comparison between tumour tissue, plasma and tumour free tissue. Kruskal-Wallis test was used in the analysis of more than two variables and Mann-Whitney U-test for analysing differences between two groups. A p-value of less than 0.05 was accepted as significant. All graphs were presented as Box-plot showing the median (horizontal line), interquartile range (boxes) and 10^{th} and 90^{th} percentiles (error bar).

Paper II

As the patient cohort was larger, a normal distribution was assumed. When comparing multiple groups, an ANOVA including Fishers' protected least significant difference (PLSD) correlation for multiple comparisons was used. Analysis of differences between tumour biopsy specimen and tumour-free bowel segments was performed with the paired t-test. A p-value of less than 0.05 was accepted as significant. All graphs were presented as Box-plots showing the median (horisontal line), the interquartile range (boxes) and the 10^{th} and 90^{th} percentiles (error bar).

Paper III-IV

The findings in paper II indicated that there might be differences in the expression between colon and the rectum. Therefore, patients in paper III and IV were analysed in relation to

tumour site (colon or rectum). The number of patients in the smallest group (rectum n=47 in paper IV and n=65 in paper III) necessitated non-parametric statistics, as the patient group was limited.

The Wilcoxon signed rank test was used for related samples and the Mann-Whitney U test was used for the analysis of independent parameters. The Cox proportional hazard method was used for uni- and multivariate analysis to determine the prognostic value of the measured markers. Hazard ratio (HR) was displayed with a 95% confidence limit (CI 95%) and p-values less than 0.05 were considered significant. The number of investigated variables in the multivariate analysis was in coherence with the number of events (deaths) in each study. The Kaplan-Meier method and Log rank test was used to compare the survival curves in relation to chosen cut-off values. Optimal cut-off values were identified by Receiver Operating Characteristics curves (ROC curves).

Graphs were presented as box-plots showing the median (horizontal line) interquartile range (boxes) and the 10^{th} and 90^{th} percentiles (error bars). Statistical analysis was carried out with SPSS 13.0 (SPSS Inc., Chicago, Illinois, U.S.A.)

Aspects on multiple testing

Multiple analyses, as performed in the papers in this thesis, are subject to the risk of mass-significance. An exception is the Fishers' PLSD test used in paper II, where multiple comparisons are taken into account. In theory, multiple testing will by chance alone make every twentieth test significant at the 5% level. Multiple comparisons can also be compensated for by using the Bonferroni correction or by adjusting the p-value to a value lower than 0.05. However, using a correction increases the risk of not discovering results through a beta-error. It remains controversial whether a Bonferroni correction is necessary or not. Using this type of correction may be considered statistically conservative. The Bonferroni method was not used in the current thesis, but an awareness of the number of analyses was a key aspect when interpreting the results. In particular, this would be applicable if the p-value was close to 0.05.

RESULTS & DISCUSSION

A prognostic marker is defined as a quality associated with prognosis or outcome, usually in terms of relative hazard of failure, whereas a predictive marker is defined as a quality that is associated with, and predicts, treatment response. This thesis addresses markers of tissue remodelling and their association with disease survival and tumour progression. Although the TNM classification is the most accurate prognostic tool available, it is insufficient in identifying all patients with poor prognosis with colorectal cancer. Therefore, identifying possible prognostic markers has been of major interest and subject to extensive research. Despite several decades of translational research and many areas showing promising results, no biochemical markers are presently in use.

However, the most significant development in recent years has been achieved in the area of predictive markers. K-ras mutation status has been associated with improved treatment response in metastatic colorectal cancer. The background of these findings are that mutations of K-ras in the corresponding kinase pathway can lead to constant activation of the epidermal growth factor receptor (EGFR), which can lead to resistance to EGF antibodies [96]. Recent results have shown that selection of patients with metastatic colorectal cancer for treatment with EGFR antibodies, (cetuximab or panitumumab), is depending on the K-ras status of the tumour. Both response to panitumumab monotherapy and improvement in progression-free survival were restricted to patients with wild-type K-ras [97]. However, the association of K-ras status and EGFR treatment in the adjuvant setting with improved survival in stage III patients is unclear.

Matrix metalloproteinases in colorectal cancer (Paper I and IV)

Several studies indicate that many MMPs are over expressed in colorectal tumours, and an increase in protein expression is correlated to an advanced Dukes' stage and to decreasing tumour differentiation [98-100]. At which time-period during tumour progression the MMPs are of most importance has been under debate. Accumulating data show the importance of MMPs in the early transition from a localised tumour to an invasive cancer [101]. As the MMPs have an essential role in degrading the basement membrane, special interest has been placed on MMP-1 and the gelatinases (MMP-2 and MMP-9), as they together are capable of degrading the collagen components of the BM. An increasing amount of evidence show that MMPs are associated with tumour progression and invasion [98-100,102,103]. Increasingly, research data indicate that the MMPs have functions other than promotion of invasion, have substrates other than components of the extracellular matrix, and that they can have a function before invasion in the development of cancer by activating growth factors and cytokines [45].
These considerations are in line with the results in paper I, as both MMP-1 protein expression in tumour tissue (Fig. 4) and MMP-2 expression in plasma (Fig. 5) were

associated with, not only increasing tumour stage (T-status), but also lymph-node metastasis in patients without distant metastatic disease (Fig. 6 and 7).

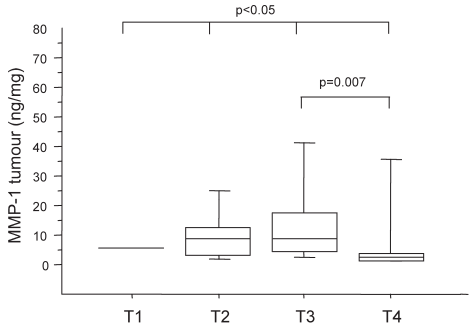

Fig. 4.
The MMP-1 protein expression in tumour tissue was significantly higher in T2 and T3 tumours compared to more advanced T4 tumours. Reprinted with permission of Springer-Verlag GmbH Heidelberg

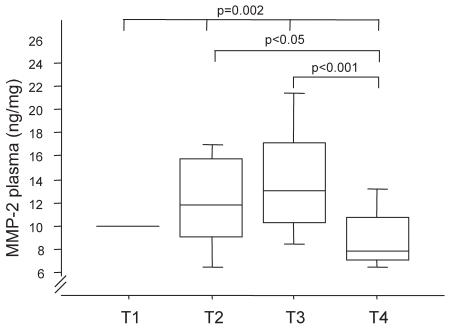

Fig. 5.
The MMP-2 protein expression in plasma was significantly higher in T2 and T3 tumours compared to T4 tumours. Reprinted with permission of Springer-Verlag GmbH Heidelberg

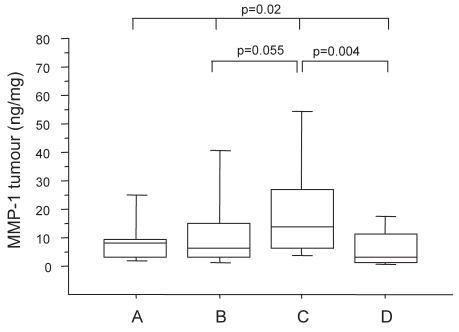

Fig. 6.
The MMP-1 protein expression in tumour tissue was significantly higher in patients with lymph node metastasis, but without distant metastatic disease. Reprinted with permission of Springer-Verlag GmbH Heidelberg.

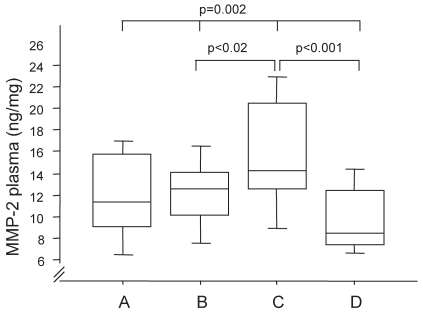

Fig. 7.
The MMP-2 protein expression in plasma was significantly higher in patients with lymph node metastasis, but without distant metastatic disease. Reprinted with permission of Springer-Verlag GmbH Heidelberg.

The association of MMP protein expression with advancing T-stage and lymph node metastasis in paper I was interesting, as the results simultaneously suggested that both low protein expression of MMP-1 in tumour tissue and low MMP-2 in plasma was associated with metastatic disease. These results are supported by Waas *et al*. who showed that low systemic MMP-2 (ELISA) and active MMP-2 expression were associated with metastatic disease [104], and this correlation was also observed in tumour tissue [105]. However, contradictory data exist and could perhaps be explained by different bioassays utilised to assess the expression [106].

The association of MMP-1 in tumour tissue and MMP-2 in plasma with tumour stage indicated that these factors could possibly be associated with survival. The finding in paper IV that MMP-1 and -9 expression in adjacent tumour free mucosa was associated with

cancer specific survival was an unexpected finding (MMP-1 mucosa; p=0.001, HR: 1.13, CI: 1.05-1.21 / MMP-9 mucosa; p<0.002, HR: 1.11, CI: 1.04-1.19).

The most encouraging result was that MMP-1 in tumour free mucosa maintained its association with CSS in patients without distant metastatic disease in both uni- and multivariate analysis. An interesting aspect of these findings was that the prognostic information that would be available in the postoperative pathology report might be available in the preoperative setting through a mucosal biopsy. The results in paper IV indicate that the MMP-1 expression in tumour free mucosa can identify patients without distant metastatic disease with high risk of poor survival to the same extent as nodal status in patients with colon cancer (Fig. 8). An interesting question would be if MMP-1 expression in tumour free mucosa was able to identify stage II patients at risk of poor survival, as current tumour classifications are insufficient in the risk evaluation in these patients. However, the number of events (deaths) in this subgroup of patients did not allow the analysis to be done from a statistical point of view.

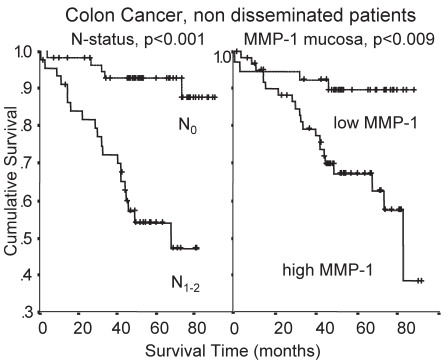

Fig. 8.
The survival curves are displayed for patients without distant metastasis, stratified by N-status (N_0/N_{1-2}) and high (>0.7 ng/mg) and low (<0.7 ng/mg) MMP-1 protein expression in tumour free tissue. Patients without lymph node metastasis (N_0) had a better outcome compared to patients with lymph node metastasis (N_{1-2}). Similar survival curves were observed when patients were stratified for the MMP-1 cut-off level. Patients with high MMP-1 expression in tumour free tissue had a significantly worse outcome compared with patients with low MMP-1 protein expression in tumour free mucosa (Log rank test).

The interplay of the tumour with the surrounding non-tumourous stroma was visualised by immunohistochemistry in paper IV, where activated fibroblasts in the tumour-free stroma in the immediate vicinity of the invasive zone, showed high MMP-1 immunoreactivity (Fig. 9). This would indicate that matrix degradation had been activated in this zone. One might therefore speculate that the capability of the tumour to mobilise the proteolytic reserves of adjacent normal intestinal mucosa, could partly determine the risk of lymphatic invasion and subsequent metastasis. Although prognostic data regarding MMP-1 protein expression is limited, semiquantitative immunohistochemistry data indicate that MMP-1 expression also in tumour tissue could be associated with tumour invasion [107] and survival [108].

As MMPs are believed to be important in the early phase of tumorigenesis and therefore a possible marker of disease progression, the lack of association between increasing tumour stage and poor survival with systemically measured MMPs has been disappointing. Results from Oberg *et al* showed limited clinical value of either MMP-2 or MMP–9 protein expression in serum for tumour staging or prognosis, although higher free MMP-2 expression in sera was associated with shorter survival time [109]. Waas *et al* found similar results [104], and in a follow-up study where gelatinase expression was compared

with CEA, systemically measured gelatinases were not able to identify patients with recurrent disease during the follow-up period [110]. These results are in line with the results presented in this thesis, as survival data showed no association of systemically measured MMP-2 and -9 protein expression with cancer specific survival. Therefore, the association of higher MMP-2 expression in plasma in patients with lymph-node metastasis seen in paper I do not seem to be of clinical relevance. The proteolytic enzymes act locally in the stroma and systemic expression might be of no or limited informative value.

Recent findings indicate that tissue concentration and immunohistochemical presence of MMP-2 protein expression in tumour tissue as well as in the surrounding stroma is associated with poor survival in univariate analysis [111,112]. The results in paper IV support these findings and there is increasing evidence that MMP-2 expression in tumour tissue is associated with poor survival in patients with colorectal cancer. However, the finding that ELISA derived MMP-2 expression in tumour tissue is associated with survival only in colon cancer is to our knowledge not previously described, although recent immunohistological data suggest that epithelial tumour expression of MMP-2 might be of differential prognostic importance in colon and rectal cancer [112].

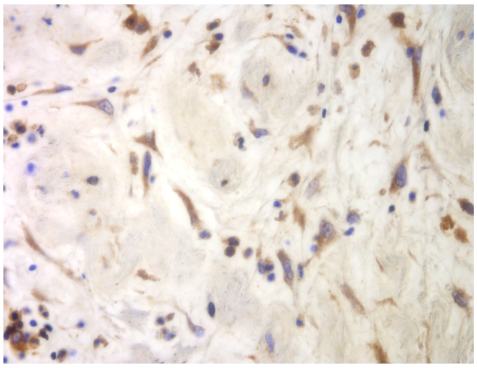

Fig. 9.
Reactive fibroblasts in the immediate vicinity of the invasive zone of the tumour are displayed by MMP-1 immunoreactivity.

The plasminogen/plasmin system in colorectal cancer (Paper III)

During the isolation and recognition of PAI-1, it was generally assumed that plasmin through the activation of plasminogen was the main pathway in which MMPs were activated and exerted their proteolytic effects during tumour invasion. This has also been the main hypothesis, namely that tumour progression and prognosis is associated with purely the degrading capacity of MMPs. In this system, the role of PAI-1 was thought to be purely inhibitory. Therefore, clinical results identifying PAI-1 expression with poor prognosis in several tumour forms, was an unexpected finding [56,62-64,113,114]. However, it has become apparent that the interplay of uPA, PAI-1 and the MMPs is complex, and that the initial assumption that the role of MMPs is limited to matrix degradation can be questioned.

Results *in vitro* have previously shown that high PAI-1 concentrations have anticancer effects [115]. Previous results on a knockout mice model have shown that endogenous PAI-1 is needed for invasion, indicating that PAI-1 can have differential mode of action in cancer development. The binding of uPA to its membrane bound receptor is shown to enhance cell adhesion. However, a complex of not only uPA/uPAR, but also uPA/uPAR/PAI-1 is needed in order to achieve cell-detachment [116]. These results

describe a possible mode of action that could partly explain the unexpected primary results, i.e. PAI-1 being associated with poor prognosis in cancer.

The results in paper III of high uPA in tumour free mucosa being associated with improved survival were unexpected. The Cox proportional regression analysis in paper III showed that higher uPA protein expression in tumour free mucosa was significantly associated with improved survival in rectal cancer (p=0.005, HR: 0.232, CI: 0.08-0.64). Also in patients without distant metastatic disease, low uPA concentrations in tumour free mucosa seemed to identify patients with increased risk of poor cancer specific survival (Fig. 10). These findings were also true for stage I and II patients, as the mucosal uPA expression below the cut-off level identified all deaths in this subgroup of patients. However, these results must be interpreted with caution, as the number of events in this subgroup of patients was low (n=8).

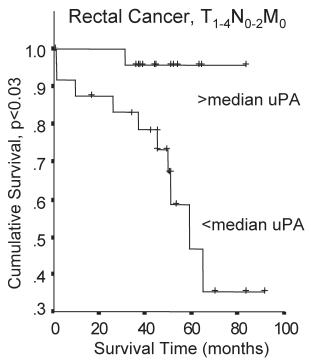

Fig. 10.
Patients were stratified by the median uPA protein expression in tumour free tissue. Patients with high uPA protein expression in tumour free tissue had a significantly better outcome compared with patients with low uPA protein expression in tumour free mucosa (Log rank test).

The biological explanation for the association of uPA in tumour free mucosa with survival is unclear. However, a higher uPA expression in tumour free mucosa might reflect less complex formation, which might be associated with decreased cell detachment. It is possible that the expression of uPA in mucosa reflects a reduced invasive potential of the tumour. On the other hand, it is possible that the uPA expression in the intestinal mucosa is a marker for a different biological phenomenon. As white blood cells are known to harbour large amounts of uPA, the improved survival in patients with high uPA concentration could be associated with immunological aspects of tumour or host immunity [41,117]. Nevertheless, in accordance with the discussion regarding the prognostic association of MMP-1 and -9 in the adjacent tumour free mucosa, it is apparent that adjacent tumour free mucosa cannot be regarded as biologically normal. These results suggest that more general and basic changes in the intestinal mucosa are present in a tumour-bearing segment, and that these changes are of differential importance in the proximal and distal large intestine.

The finding that PAI-1 in plasma was associated with cancer specific survival is consistent with previous reports [71]. Our results indicate that high PAI-1 in tumour tissue and plasma is associated with poor survival and that the inverse association pertains to uPA expression in tumour free mucosa. As shown in paper III, PAI-1 protein expression in plasma correlated to metastatic disease in patients with rectal cancer ($p<0.0001$, $r=0.524$, $n=62$), and was significantly higher ($p<0.0001$) in rectal cancer patients with distant

metastatic disease. This finding is consistent with the results of the Cox proportional hazard analysis, as the association of PAI-1 in plasma with survival was abolished when studied together with M-stage (multivariate analysis: M-stage: p<0.0001, HR: 20.59, CI: 5.20-81.49 / PAI-1: p=0.72, HR 1.15, CI: 0.053-2.53). This suggests that the systemic expression of PAI-1 is associated with distant metastatic disease. As PAI-1 has been reported to act as an acute phase reactant [118], the association with poor survival might be an indication of a more generalised systemic response to distant metastatic disease rather than the tumour itself. Due to the exclusion of patients that received radiotherapy, the number of patients with metastatic disease was increased in rectal cancer group. This could have strengthened the prognostic association seen in rectal cancer compared with colon cancer. However, no association of uPA and PAI-1 expression with survival was seen in colon cancer, indicating a different tumour environment in colon and rectal cancer.
It is conceivable that PAI-1 could be used in the follow-up of patients with rectal cancer after surgery with a curative intent in order to identify patients with recurrent metastatic disease. However, conclusions regarding this aspect cannot be addressed in the context of this thesis, but further studies addressing this issue seem warranted.

Transforming growth factor-β1 in colorectal cancer (paper II and IV)

There is increasing evidence that TGF-β1 has several functions, and exerts both tumour-suppressive and oncogenic effects [37], and that TGF-β1 is strongly associated with the regulation of extracellular remodellation.

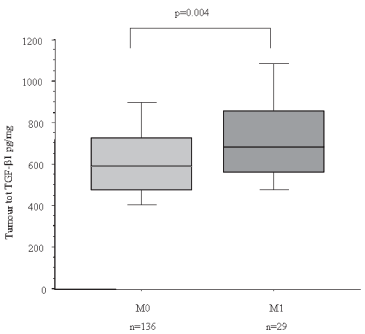

Fig. 11.
Significantly higher TGF-β1 expression in tumour tissue was seen in patients with colorectal cancer and distant metastatic disease (M_1) compared with patients without distant metastasis (M_0). Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc

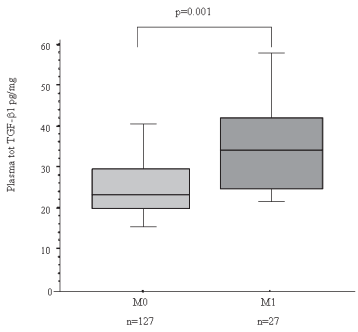

Fig. 12.
Significantly higher TGF-β1 expression in plasma was seen in patients with colorectal cancer and distant metastatic disease (M_1) compared with non-disseminated patients (M_0). Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc

In paper II, increasing T-stage was positively correlated with higher TGF-β1 protein expression in tumour tissue (p=0.001, r=0.297). As the correlation could be due to the fact that T4 tumours are frequently associated with metastatic disease, a separate analysis was done, excluding patients with distant metastatic disease. However, the correlation remained

positive (r=0.237, p=0.001), indicating also a localised role of TGF-β1 in tumour invasion. Distant metastatic disease influenced total TGF-β1 protein expression, and total TGF-β1 protein expression in both tumour tissue and plasma was significantly higher in patients with metastatic colorectal cancer compared to patients with non-metastasising disease (Fig. 11 and 12)**.**

There are previous reports of higher TGF-β1 plasma and serum protein expression in patients with metastatic disease [119,120], which is further supported by the results in paper II. These studies indicated that TGF-β1 protein expression, although most predominantly expressed in patients with metastasising disease, also might be dependent of the local invasiveness of the tumour, as expression correlated with increasing T-status, (Fig. 13).

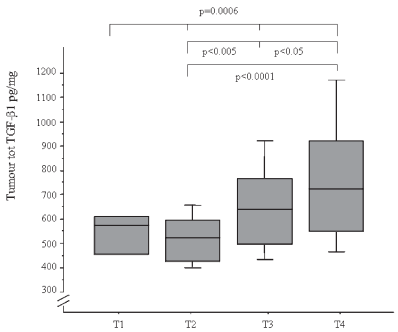

Fig. 13.
TGF-β1 protein expression in tumour tissue was significantly associated with increasing T-status in patients with colorectal cancer. Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc

Although TGF-β1 is tightly linked to MMP regulation, our results indicated a separate mode of action for these markers during tumour progression in colorectal cancer.

Higher expression of TGF-β1 in patients with metastatic disease was supported by the results in the Cox proportional hazard analysis, which indicated that TGF-β1 expression in tumour tissue was weakly associated with poor survival in both colon and rectal cancer. TGF-β1 concentration in plasma was significantly associated with CSS in rectal cancer patients. This association was not maintained in multivariate analysis due to the strong association to metastatic disease. The results in paper II suggested that local TGF-β1 expression in tumour tissue could be of importance in evaluating tumour progression and subsequent worse survival. These findings were not supported by survival analysis, as no association with CSS and TGF-β1 expression was seen in patients without distant metastatic disease. However, the study by Tsushima *et al* where 5-year-survival was evaluated, high preoperative TGF-β1 protein expression in plasma was strongly predictive of recurrent disease manifested as liver metastasis after curative resection of colorectal cancer [121]. These results could not be confirmed in paper IV. A possible explanation is the different endpoints utilised. In the present studies CSS was evaluated in contrast to disease free survival used by Tsushima *et al*. Additionally, the exclusion of non-irradiated patients or a shorter follow-up period for the patients included in this thesis could partly explain the different results.

Aspects on the adjacent normal intestinal mucosa

The observation that the expression of MMP-1 and MMP-9 in tumour free intestinal mucosa was associated with survival was an intriguing concept. However, a similar observation has previously been reported in subjects with gastric cancer [122]. An important question would be if these changes in adjacent, apparently normal mucosa precede tumour development or if they are a function of the presence of the tumour. The present thesis does not allow any conclusions in this matter. However, the results indicate that the presence of a tumour in the colon or rectum can be associated with more widespread biological changes in the large intestine.

The immunohistochemical analysis in paper IV indicated that morphological differences in staining pattern in tumour free mucosa between colon and rectal cancers can be present. This could suggest a different biological tumour environment in colon and rectal cancer, supporting the divergent prognostic impact of MMP-1 in adjacent tumour free mucosa observed for these two types of cancer. The results from paper III also suggest that colon and rectal cancers could be regarded as two separate tumour forms, as the differential prognostic association with survival of uPA expression in mucosa was present. There are several reports regarding different cellular and expressional pattern in the adjacent normal mucosa in patients with a colon or rectal carcinoma [123-125]. Paper II indicated that TGF-β1 expression in tumour free mucosa in patients with colon cancer differs compared to rectal cancer patients. Recent results show two different gene expression patterns in the normal mucosa of the large intestine [126]. One pattern is consistent with the midgut-hindgut embryonic origin, and another pattern displaying a gradual change in transcript of multiple genes along the large intestine.

In summary, different molecular expressional profiles are likely to be present in the apparently normal mucosa of the proximal and distal colon. In addition, the adjacent normal mucosa expresses properties that have impact on the prognosis in colon and rectal cancer. Interestingly, this thesis also suggests that measured factors in normal mucosa of a tumour bearing segment that are capable of matrix degradation, are differentially associated with survival in colon and rectal cancer.

Expressional and prognostic differences in tumour tissue in the proximal and distal large intestine

Results in paper II indicated that TGF-β1 protein expression in tumour tissue was higher in colon cancers compared to rectal cancers (Fig. 14). Expressional data on both protein and mRNA level support these data, indicating a different tumour biology in the proximal and distal colon [127,128]. Results also suggest that uPA has a differential expression in the proximal and distal large intestine [129]. The concept that the colon and rectum represent two different entities from a tumour biology point of view is supported by evidence that two different genetic mechanisms, microsatellite instability (MSI) and chromosomal instability (CIN) contribute unevenly to the carcinogenesis in the different parts of the lower GI-tract [130,131]. The prognostic differences in the present study could possibly be

explained by these underlying mechanisms in the colon and the rectum. The effect of the exclusion of irradiated patients could have influenced results. However, the comparison to irradiated patients included in other studies during the same period indicate, that no T-status migration is present between irradiated and non-irradiated rectal cancer patients (unpublished data). The reason for this distribution is unclear and might indicate a suboptimal selection of patients for preoperative radiotherapy. It is therefore reasonable to assume that the results obtained could be valid for all patients with rectal cancer.

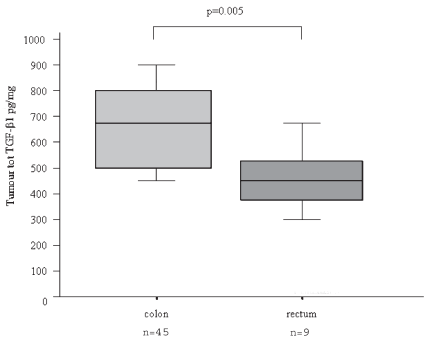

Fig. 14.
TGF-β1 protein expression in tumour tissue was significantly higher in patients with colon cancer compared with patients with rectal cancer. Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc

Aspects on prognostic information in different tissue compartments

Results included in this thesis indicate that the association of systemically measured factors with survival is due to their strong correlation with metastatic disease. These findings might reflect, perhaps not a specific proteolytic mechanism, but rather a generalised response to metastatic disease and inflammation. Previous research demonstrates that many markers are associated with metastatic disease, and that the association with disease survival decreases in the subgroup of patients without presence of distant metastatic disease [32].

However, in patients without metastatic disease, tumour free mucosa was most strongly associated with CSS. One would have expected that the essential prognostic information should have been found in tumour tissue. The reason for this might be found in tissue processing. The homogenisation of tissue has been commonly used in translational research. It is however conceivable that much of the prognostic value is diluted by this process, as one can assume that the majority of colorectal tumours are very heterogenic. As adjacent tumour free tissue had the most bearing on the postoperative prognosis in our studies, it could be hypothesised that a homogenic tissue compartment, such as tumour free mucosa or plasma, might give more solid prognostic data.

In the future, it might be more appropriate to consider micro dissection as a tool for ECM tissue sampling, as specific stromal structures without contamination of tumour cells, can be chosen for further analysis. However, this would limit available analysing techniques, as protein detection requires a substantial amount of tissue. In principal, only Real Time Polymerase Chain Reaction (RT-PCR) techniques would probably be adequate investigational tools today, as a small amount of tissue is needed for mRNA evaluation.

Tissue remodelling components and their biological role in tumour progression: Summary and conclusion

In summary, TGF-β1 and PAI-1 protein expression indicate similar features in relation to tumour progress in colorectal cancer, as they were associated with distant metastatic disease. Both markers were associated with known pathological staging classifications and were over-expressed in both tissue and plasma in rectal cancer patients with metastatic disease. However, their prognostic impact seems limited in patients without distant metastatic disease.

The results in this thesis seem to support the assumption that MMPs are of importance, especially in the early stage, of tumour invasion and progression. Results in paper I and IV suggested that MMPs are over-expressed in the invasive period of the tumour when lymph node metastasis occur. There are also indications of lower MMP expression in all tissue compartments (mucosa, tumour and plasma) in patients with distant metastatic disease, indicating that once dissemination has occurred, the local expression might no longer be of importance.

Although it seems logical that MMPs are expressed locally during tumour invasion, the association of MMP-1 expression in adjacent tumour free mucosa with cancer specific survival was unexpected. This also means that prognostic information would be accessible in the preoperative setting through a mucosal biopsy. However, the biological background for the differential association of tissue remodelling factors with CSS in colon and rectal cancer is unclear and needs to be further understood.

Lastly, the findings in this thesis suggest a different view on the role of apparently macroscopically normal mucosa in the tumour-bearing segment, as it seems evident that important changes in the microenvironment, even remote from the tumour, are present.

Future perspectives

Validation of MMP-1 expression in tumour free mucosa as a future prognostic marker in colon cancer would be valuable. Further evaluation of MMP-1 expression in mucosa in the adjacent tumour free bowel in relation to the increasing distance from the primary tumour could give important insight to the regulation of the tumour microenvironment in colorectal cancer. It is also conceivable that the prognostic strength for CSS is variable in relation to the distance from the tumour, and it would be important to evaluate if the prognostic information is reduced with increasing the distance from the tumour. This study design might give the opportunity to evaluate if changes in the microenvironment of the tumour are present regardless where the intestinal mucosal biopsy is taken.

Further investigation of the matrix in the desmoplastic stroma and the invasive zone of the tumour seem warranted. Preferable techniques would be the use of micro-dissection, as this would facilitate the analysis of specific host matrix components. This could perhaps

eliminate confounding regulatory factors generated by the tumour, and aid the specific evaluation of the microenvironment and extracellular matrix surrounding tumour tissue.

One of the most important questions not answered by this thesis is if factors capable of matrix degradation, could aid in the identification of stage II patients with high risk of poor survival. These patients are problematic to study, as there are fewer deaths in this subgroup of patients, necessitating large cohorts. These studies would preferably be performed as prospectively controlled, multi-centre trials.

The fundamental principle of the pre- and postoperative classification of a tumour is to optimise neo-adjuvant, adjuvant and surgical treatment. Our results indicate that a mucosal biopsy has a similar prognostic association with survival as N-status. However, N-status information cannot be obtained until the histopathology report is available after surgery. The information obtained in a mucosal biopsy could therefore provide the argument to offer neo-adjuvant therapy to high-risk patients and to optimise surgical technique in order to increase the likelihood of curative surgery for these patients.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Kolorektal cancer är den vanligaste formen av cancer i Sverige efter de könsbundna tumörformerna (prostata cancer för män och bröstcancer för kvinnor). Trots betydande medicinska och kirurgiska framsteg är dödligheten i kolorektal cancer mellan 40-50%. Prognosen är beroende av hur djupt tumören infiltrerar i tarmväggen, samt huruvida tumören utvecklat lymfkörtelspridning i tarmkäxet. Den grundläggande tumörklassifikationen som är baserad på patologens preparatundersökning är otillfredsställande och behovet av prognostiska faktorer som ytterligare skulle förbättra diagnostiken av patienter med risk för cancerspecifik död är stort.

För att kunna tillväxa och sprida sig måste tumören bryta igenom anatomiska barriärer, såsom tarmens muskellager och underliggande stödjevävnad. Avhandlingens hypotes var att faktorer som deltar i denna nedbrytningsprocess såsom matrix metalloproteinaser (MMPs) och dess aktivatorer och inhibitorer (urokinase plasminogen activator, (uPA), plasminogen activating inhibitor-1 (PAI-1)), går att mäta i tumörvävnad, tumörfri vävnad samt i blod och att dessa faktorer skulle kunna relateras till tumörstadium och/eller kunna prognostisera cancerspecifik död.

Patienter med kolorektal cancer inkluderades under tidsperioden 1999-2004. I samtliga fyra delarbeten togs vävnadsbiopsier från tumörvävnad, närliggande tumörfri slemhinna samt blod under det kirurgiska ingreppet. Proteinnivåer av vävnadsremodellerande proteaser analyserades.

Vi fann signifikant högre koncentrationer av samtliga analyserade markörer i tumörvävnad jämfört med i tumörfri slemhinna. Detta indikerar en aktiv ombyggnad i tumören. Såväl reglerande plasminogen associerade faktorer som proteolytiska enzymer var associerade med den diagnostiserade postoperativa tumörklassifikationen.

Våra resultat indikerar att faktorer som deltar i vävnads remodellering är associerade med cancerspecifik död, samt att denna association skiljer sig mellan koloncancer och rektalcancer. Ett oväntat fynd var att den tumörfria tarmslemhinnan avspeglade tumörprogression och att markörerna (MMP-1 i koloncancer och uPA i rektalcancer) här förefaller vara associerade med cancerspecifik överlevnad.

Resultaten i denna avhandling talar för att man vid evalueringen av biomarkörer för kolorektal cancer bör ta hänsyn till tumörens lokalisation i grovtarmen. Resultaten indikerar även att information angående patientens prognos, som nu är beroende av patologutlåtandet efter det kirurgiska ingreppet, skulle kunna vara tillgängligt redan innan det kirurgiska ingreppet genom att analysera en provbit från den tumörnära, men makroskopiskt tumörfria tarmslemhinnan. Denna information skulle kunna identifiera patienter med ökad risk för cancerspecifik död, samt även ge förutsättningar för att innan operation påbörja behandling för dessa selekterade patienter.

ETHICAL ASPECTS

The study was approved by the Local Ethics Committee (Application L019-99, Gothenburg University) and informed consent was obtained from all participating patients.

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