Prognostic factors in colorectal cancer
Aspects of tumour dissemination
by
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Abstract:
Colorectal cancer (CRC), the most common gastrointestinal cancer, causes around 2500 deaths annually in Sweden. Tumour resection is still the only curative treatment, but surgery is successful in only about half of the patients. The best prognostic factor so far is the tumour stage according to the TNM classification system which is based on the perioperative findings of the extent of the tumour growth. Adjuvant chemotherapy has been shown to reduce the number of deaths with about one third in the selected group of patients with metastatic disease in the regional lymph nodes. Among patients having surgery for tumours in more favourable stages, still some will die from cancer that might have been cured by adjuvant chemotherapy. We have therefore looked for better prognostic factors to improve the selection of patients for adjuvant chemotherapy treatment.

In our first paper, we analysed the prognostic impact of immunohistologic detection with cytokeratin 8/18 of disseminated tumour cells in regional lymph nodes among 147 patients without any detected tumour cells at routine examination. We observed tumour cells in lymph nodes from 32% of the patients, but their presence had no prognostic impact on survival.

In the next study, we evaluated preoperative serum levels of the metalloproteinases MMP-2 and MMP-9 and their inhibitors TIMP-1 and TIMP-2, and we found elevated levels in cancer patients compared to healthy controls. However, large overlapping ranges between serum levels in patients and controls reduced the clinical benefit of the test.

In paper three, we studied the metastatic tissue in lymph nodes from 93 patients regarding the number of immune cells showing positivity for CD8, CD45R0 and CD68. High numbers of these immune cells correlated to a favourable prognosis.

In paper four, we developed a method using real-time quantitative RT-PCR for detection of CEA mRNA in regional lymph nodes with microscopically normal appearance as a marker for disseminated tumour cells. Quantitative RT-PCR presented as a highly sensitive and specific method. The clinical impact of detected CEA mRNA in lymph nodes according to survival remains to be evaluated in further studies.

In summary, our studies aimed to identify methods for improved tumour staging in CRC indicate, that real-time quantitative RT-PCR for CEA seems to be a more sensitive method than immunohistologic detection of CEA-positive cells for early detection of disseminated disease in the regional lymph nodes.

Key words: Colorectal neoplasms, prognosis, lymph node metastases, disseminated tumour cells, cytokeratins, micrometastases, metalloproteinases, immune cells, real-time quantitative RT-PCR, carcinoembryonic antigen
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COVER PICTURE:
Section of a regional lymph node with cytokeratin 8/18 positive tumour cells.
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ABBREVIATIONS

APC adenomatous polyposis coli gene
CEA carcinoembryonic antigen
CK cytokeratin
CRC colorectal cancer
DCC deleted in colon cancer gene
EGF epidermal growth factor
ELISA enzyme linked immunosorbent assay
FAP familial adenomatous polyposis
FGF fibroblast growth factor
HNPCC hereditary nonpolyposis colorectal cancer
ICC immunocytochemistry
IHC immunohistochemistry
IL interleukin
mAb monoclonal antibody
MASA mutant allele specific amplification
MM micrometastases
MMP matrix metalloproteinases
MRD minimal residual disease
MSI microsatellite instability
PBMC peripheral blood mononuclear cell
qRT-PCR quantitative reverse transcriptase-polymerase chain reaction
RIGS radioimmunoguided surgery
TGF transforming growth factor
TIMP tissue inhibitor of metalloproteinases
TNF tumour necrosis factor
TS thymidylate synthase
VEGF vascular endothelial growth factor
ABSTRACT
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Aspects of tumour dissemination
Åke Öberg, Departments of Surgical and Perioperative Sciences, Surgery; Clinical Microbiology, Immunology; Medical Biosciences, Pathology, Umeå University, Sweden

Colorectal cancer (CRC), the most common gastrointestinal cancer, causes around 2500 deaths annually in Sweden. Tumour resection is still the only curative treatment, but surgery is successful in only about half of the patients. The best prognostic factor so far is the tumour stage according to the TNM classification system, which is based on the perioperative findings of the extent of the tumour growth. Adjuvant chemotherapy has been shown to reduce the number of deaths with about a third in the selected group of patients with metastatic disease in the regional lymph nodes. Among patients having surgery for tumours in more favourable stages, still some will die from cancer that might have been cured by adjuvant chemotherapy. We have therefore looked for better prognostic factors to improve the selection of patients for adjuvant chemotherapy treatment.

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In the next study, we evaluated preoperative serum levels of the metalloproteinases MMP-2 and MMP-9 and their inhibitors TIMP-1 and TIMP-2, and we found elevated levels in cancer patients compared to healthy controls. However, large overlapping ranges between serum levels in patients and controls reduced the clinical benefit of the test.

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In summary, our studies aimed to identify methods for improved tumour staging in CRC indicate, that real-time quantitative RT-PCR for CEA seems to be a more sensitive method than immunohistologic detection of CEA-positive cells for early detection of disseminated disease in the regional lymph nodes.

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Cancer i tjock- och ändtarm är den vanligaste elakartade buktumören i Sverige. Årligen insjuknar ca 5000 personer, varav en hälften dör av sjukdomen. Kirurgi är den enda behandling som kan erbjuda bot, men trots operation där all synlig tumörvävnad tas bort återinsjuknar omkring en tredjedel av patienterna. Den hittills bästa metoden att förutsäga återinsjuknande är den mikroskopiska undersökningen, där tumörens utbredning i tarmvägg samt spridning till lymfkörtlar och andra organ bedöms. Patienter med påvisad tumörspridning till lymfkörtlar får återfall i omkring hälften av fallen. Denna högriskgrupp av patienter erbjuds idag tilläggsbehandling efter operationen i form av upprepade kurer med cellgift. Detta har visat sig medföra en minskning av antalet återfall med cirka en tredjedel. Då närmare hälften av patienterna i denna grupp är botade av enbart kirurgi innebär det sammantaget att ca 80% av patienterna får behandlingen utan positiv effekt. Även vissa patienter med tumörväxt begränsad till tarmväggen återinsjuknar och också denna grupp av patienter skulle kanske kunna vara hjälpta av tilläggsbehandling, men med dagens urvalsmetoder erbjuds de ej detta. Dessa studier har haft som mål att försöka finna metoder att på ett bättre sätt välja ut patienter för tilläggsbehandling.

I arbete I användes färgning med antikroppar mot en tumörcells markör (cytokeratin 8/18) för att möjliggöra identifiering av enstaka tumörceller i lymfkörtlar. Tumörceller påvisades hos 32% av patienterna som bedömts ha normala lymfkörtlar vid rutinmässig mikroskopundersökning, men förekomst av tumörceller påvisade med denna metod kunde ej förutsäga återfall av tumörsjukdom.

I studie II analyserades blodprov tagna före operationen på halten av vävnadsnedbrytande enzym (metalloproteinaser och deras hämmare), som anses betydelsefulla för tumörens möjlighet att sprida sig ut i omgivande vävnad. Tumörpatienter hade som grupp förhöjda värden jämfört med en frisk kontrollgrupp. Värdena överlappade dock varandras åt så mycket att någon klinisk nytta av blodprovet för att förutsäga spridning av tumören hos den enskilda patienten ej förelåg.

I arbete III studerades betydelse av antalet vita blodkroppar/immunceller i tumörvävnad som spridit sig till lymfkörtlarna. De patienter med högre antal immunceller hade en bättre chans till överlevnad än patienter med lågt antal celler. Det är tänkbar att den bättre chansen att överleva hos den första gruppen beror på en immunsvarsreaktion mot tumörceller.

ORIGINAL PAPERS

This thesis is based on the following papers, referred to in the text by their roman numerals:

I. Öberg Å, Stenling R, Tavelin B, Lindmark G.

II. Öberg Å, Höyhtyä M, Tavelin B, Stenling R, Lindmark G.

III. Öberg Å, Samii S, Stenling R, Lindmark G. Different occurrence of CD8+, CD45R0+ and CD68+ immune cells in regional lymph node metastases from colorectal cancer as potential prognostic predictors.

Detection of occult tumour cells in lymph nodes of colorectal cancer patients using real-time quantitative RT-PCR for CEA mRNA. Submitted.

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INTRODUCTION

Colorectal cancer

General characteristics

The incidence of colorectal cancer (CRC) varies much with high incidence in industrialized societies like USA, Europe, and Australia, and low incidence in rural developing countries. The etiology of the disease is obscure, but life style factors like the use of alcohol and cigarettes as well as dietary factors like low-fibre and high fat intake, have been proposed. Most cases of CRC are sporadic, but 10-15% of the patients have predisposing hereditary germline alterations.

CRC, the most common gastrointestinal cancer in Sweden, is after prostate and breast cancer the third most common cancer contributing to about 12% of the total incidence of cancer. During the last four decades the incidence of both colon and rectal cancer has increased among both males and females, resulting in a lifetime risk of developing CRC of 9% for males and 7% for females. However, the mortality has been stable over time.

In Sweden, there is an annual incidence of approximately 50 CRC cases per 100 000 inhabitants, which corresponds to almost 5000 new CRCs diagnosed and 2500 deaths related to the disease every year. Colon cancer is evenly distributed according to gender, but rectal cancer is more common among males.

Most patients are elderly at diagnosis with a median age of 70-74 years. Approximately a third of the tumours are located in the rectum, 25% in the sigmoid colon, and 40% in the remaining colon. More than 95% of the tumours are adenocarcinomas derived from the epithelium, whereas carcinoids, lymphomas, and leiomyosarkomas are rare.

The progression from normal mucosa to cancer is believed to include the formation of adenomas, and a successive accumulation of genetic changes postulated in the adenoma-carcinoma sequence of Vogelstein et al. 1988.

A second pathway for tumour progression has been discovered requiring inactivation of the DNA mismatch repair system. A hereditary defect inactivating this repair system causes the hereditary nonpolyposis colorectal cancer (HNPCC), which constitutes 1-5% of all CRC. There are also other less common hereditary syndromes like familial adenomatous polyposis (FAP) resulting in multiple adenomatous polyps which untreated progress into cancer already at a mean age of 40 years.

The most common sites for metastatic disease in CRC are the regional lymph nodes and the liver. This may be due to the anatomical location in combination with the fertile environment provided in the liver for tumour growth. Experimental models have shown that most tumour cells are trapped in the capillary beds of the organ downstream from the tumour. The occurrence of circulating tumour cells in the peripheral blood is, however, not uncommon even in early tumour stages. The finding of lung metastases without concomitant liver metastases is described in 2 to 4% of patients, who relapse in CRC after a potentially curative resection. This observation indicates alternative routes for metastases.

The overall five years survival rate in CRC is about 50%, and the only treatment for cure is surgery with complete removal of the tumour. Approximately 70% of the patients undergo surgical resection with curative intent. Unfortunately, even when all visible tumour has been removed, approximately one third of the patients die from recurrent disease within 5 years. This suggests, that these patients have occult disseminated cancer cells at the time of primary surgery. Neoadjuvant radiotherapy has reduced the number of local recurrences from rectal cancer and also improved the survival rate. There have also been some studies concerning the effect of adjuvant chemotherapy in colon cancer patients with metastatic disease in their regional lymph nodes. These show a reduction in the mortality by about one
Figure 1. Incidence — and mortality — rates in colorectal cancer in the Northern Swedish Health Care Region 1959-1996 (age standardized per 100,000, by sex)

Mutation or loss of

APC
(5q)

DNA hypomethylation

Loss of

DCC
(18q)

Loss of

p53
(17p)

Other Alterations

Normal Epithelium → Early Adenoma → Intermediate Adenoma → Late Adenoma → Carcinoma → Metastasis

Figure 2. A genetic model for tumour progression (location on chromosome) Modified from Fearon and Vogelstein, 1990
However, half of these patients are cured by surgery alone, which means that over 80% of the patients receive treatment without any effect on survival. Instead the treatment with adjuvant chemotherapy is costly and also associated with negative side effects. There are also a substantial number of patients without metastatic disease in their lymph nodes, who get recurrences and die from the disease. These patients may also benefit of adjuvant chemotherapy, but today there is no method available for selecting them to therapy. The overall purpose of these studies was to explore new methods for selecting patients to adjuvant therapy.

**Tumour stage**
There has been much research in CRC to identify prognostic factors of outcome of the disease as well as predictive factors of response to therapy. The first, and by tradition most widely used classification system, was introduced by Dukes already in 1932. It was originally designed for rectal cancers, but later applied also to colon cancers. The basis of the Dukes’ classification system is the extent of the tumour penetration into the bowel wall, involvement of the regional lymph nodes, and the presence of distant metastases in combination with the perioperative findings. The classification into Dukes’ stages A, B, C, and D corresponds to five year survival rates of >85%, 50-80%, 30-50%, and <5%, respectively. Most recurrences and deaths occur during the first two years after surgery. The results for 471 patients with CRC treated at the Department of Surgery, Umeå University Hospital, between 1987-1994 are presented in Figure 3.

In 1977, the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) published the first edition of the TNM classification system, which today is the most refined and recommended staging system. The basis of this system is, like in the Dukes’ classification, the clinicopathologic perioperative findings using T (tumour extent), N (lymph node status), and M (presence or absence of metastases). The most recent modified 5th edition of the AJCC cancer staging manual was presented in 1997. The relation between the two staging systems and the corresponding survival rates is shown in Table 1. Several other well studied factors are also recommended by AJCC to be recorded as part of good clinical practice, and they are listed as follows.

**Residual tumour classification**
The strongest prognostic factor before treatment is the TNM stage, and after treatment the residual tumour status (R classification). It is based on clinical and pathologic findings like the results of treatment locally, presence of distant metastases, and radical margins of the specimen. In general, long-term survival can be expected only after R0 resection (resection without residual tumour). Other R-classifications are R1: microscopic residual tumour, R2: macroscopic residual tumour, and RX: the presence of residual tumour cannot be assessed.

**Tumour grade**
Histopathologic grading according to the extent of the preserved glandular structures has, in numerous studies, been shown to be of independent prognostic significance. However, the grading of CRC has been difficult to standardize. Therefore, a two-tiered system has been proposed as low grade (well and moderately differentiated, >50% of the tumour cells showing gland-like structures), and high grade (poorly and undifferentiated, <50% glandular struc-
TNM Staging  Dukes’ Staging

TX Primary tumour cannot be assessed  A Tumour has not penetrated beyond muscularis propria
T0 No evidence of primary tumour  B Tumour has penetrated beyond muscularis propria, no nodal involvement
Tis Carcinoma in situ; intraepithelial or invasion of the lamina propria

T1 Tumour invades the submucosa  A Tumour has not penetrated beyond muscularis propria
T2 Invasion but no penetration through muscularis propria

T3 Penetration through muscularis propria and into the subserosa or pericolic/rectal fat but not to visceral peritoneum or other organs  B Tumour has penetrated beyond muscularis propria, no nodal involvement

T4 Invasion of other organs or involvement of the visceral peritoneum

N0 No nodal involvement  C Lymph node involvement
N1 1-3 pericolic/rectal nodes involved
N2 >3 pericolic/rectal nodes involved
N3 any lymph node along named vascular trunk and/or metastasis to apical node(s)

MX presence of distant metastases cannot be assessed
M0 no distant metastases
M1 distant metastases  D distant metastases or local spread to adjacent organs

Stage grouping

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<td></td>
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<td>any T</td>
<td>N3</td>
</tr>
<tr>
<td>Stage IV</td>
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Table 1. The relation between TNM and Dukes’ staging systems.
tures) in order to reduce the interobserver variability.22

**Tumour border**
The growth pattern of the advancing edge (i.e. the tumour border), which is to be described as "infiltrating" or "pushing", has been shown to be of prognostic significance independent of tumour stage.26,27 ("Infiltrating" associated to worse prognosis). However, a great interobserver variability by evaluation of pathologists has been shown.28

**Tumour invasion of vessels and nerves**
Invasion of tumour cells into venous and/or lymphatic vessels has been shown to have an adverse impact on prognosis.29 Perineural invasion is also a negative prognostic factor.30 The disparate results in different studies may be due to factors such as the small number of patients studied, difficulties to recognise vessels of different types, as well as tumour heterogeneity.22

**Radial margins**
In rectal cancer, the distance from the tumour closest to the radial margin of the non-peritonealised surface of the specimen (<1mm) has been shown to be of great importance when predicting local recurrences.31,32 A recent study shows an increased risk already when margins are ≤2mm.33 The same impact of radial margins is plausible also in colon cancer even if studies are lacking.

**Peritoneal involvement**
The presence of tumour cells close to or at the serosal surface has been shown to be associated with negative prognosis.34 The difficulties in the histopathologic assessment of such conditions are however substantial, why cytological examination using various techniques on peritoneal lavage fluid have been tested.35 Malignant cells in lavage have been found in 15-30% already in tumour stage I and II.35,36 The detection of malignant cells in the peritoneal cavity was shown to be associated with worse outcome.35-37

**Carcinoembryonic antigen (CEA) and other serum tumour markers**
CEA, first described in 1965, is probably the most extensively studied serum tumour marker in adenocarcinoma.39 CEA is a highly glycosylated protein containing approximately 50% carbohydrate with a molecule weight of nearly 200 kDa. Initially CEA was hypothesised to be an oncofetal antigen, expressed during fetal life, absent in adults and re-expressed in cancers, but today we know that CEA is expressed in normal adult tissue.40 The human CEA gene family belongs to the immunoglobulin superfamily and consists of 29 genes on chromosome 19q, of which 18 are expressed.40 Seven of the genes belong to the CEA subgroup and 11 genes to the pregnancy specific glycoprotein (PSG) subgroup. The remaining 11 genes are pseudogenes, i.e. nonfunctional DNA. The CEA subgroup members are all attached to the cell surface membrane while the PSGs are secreted molecules.

CEA is expressed in small amounts in many normal adult tissues, and especially on the surface of certain epithelial cells, mainly along the alimentary tract and particularly on the columnar epithelial cells in the colon.40 The production and release of CEA is substantial and about 50-70 mg of CEA can be recovered in normal feces during one day. The expression of CEA is retained or even elevated in CRC. In contrast to the normal secretion of CEA into the colon lumen the cancer cells express and secrete CEA from the whole cell surface and CEA can via the intercellular spaces reach and be detected in serum.40

Specific detection of CEA at both the protein and mRNA level has been complicated by the great homology with the closely related genes in the CEA subgroup. In normal epithelial colon cells apart from CEA also non-specific cross-
reacting antigen/NCA (CEA cell adhesion molecule-6/CEACAM6 according to new nomenclature\textsuperscript{41}), biliary glycoprotein/BGP (CEACAM1) and CEA gene family member-2/CGM2 (CEACAM4) are expressed.\textsuperscript{42} Furthermore, BGP, NCA, CGM1 (CEACAM3) and CGM6 (CEACAM8) are expressed in immune cells. It is vital for detection of tumour cells in blood, lymph nodes or bone marrow using CEA (CEACAM5) or CEA mRNA as a marker that there is no cross-reactivity with these proteins.\textsuperscript{40}

The normal function of CEA is unclear, but functions like an intercellular adhesion molecule, and importance for the innate immune defence against microbial attacks in the gastrointestinal tract have been postulated.\textsuperscript{40}

Increased serum CEA levels are noticed in a variety of cancers, particularly cancers in the gastrointestinal tract, ovary, lung, and breast\textsuperscript{43} but, unfortunately, also among some healthy individuals, especially smokers. In CRC, elevated levels have been reported from 23\% of the patients with tumours in Dukes’stage A increasing to 90\% in patients having Dukes’ stage D tumours.\textsuperscript{44,45} These limitations in both specificity and sensitivity have reduced the use of serum CEA in screening.\textsuperscript{46,47} Serum CEA has also been used in follow-up after surgery for earlier detection of recurrences\textsuperscript{48} and as an indicator for second-look surgery.\textsuperscript{49,50} The clinical benefit of this in terms of survival remains to be proven. However, a preoperative serum level of CEA >5.0 ng/mL has in several studies been shown to have an adverse effect on survival independent of tumour stage.\textsuperscript{45,51}

Other serum tumour markers, like CA19-9, CA50, CA242, and TPA have failed to be of any substantial benefit in the clinical practise.\textsuperscript{45}

**Additional tumour related prognostic genetic factors**

Many different tumour related factors and molecular markers have been studied, unfortunately in many small and retrospective studies with different and nonstandardised techniques.\textsuperscript{22} The pronounced tumour heterogeneity further contributes to the sometimes contradicting results. Heterogeneity in tumour cell proliferation with differences between the luminal and invasive margins has been shown.\textsuperscript{52} In another study Lindforss et al.\textsuperscript{53} showed, that loss of heterozygosity (LOH) of p53 and DCC, when four biopsies of each tumour were analysed, varied in a random fashion within the tumours. They have also shown in studies with multiple (up to 50) biopsies from each tumour, the presence of mutated DCC, k-ras, and p53 in all five examined tumours.\textsuperscript{54} Baisse et al.\textsuperscript{55} have also found a significant genetic heterogeneity, whereby they propose that the use of genetic markers for prognosis should be reconsidered. A selection of these factors and molecular markers are briefly discussed in the following.

**Microsatellite instability (MSI)**

Mutations in the DNA mismatch repair genes in HNPCC colon cancer are also present in 15-20\% of tumours from patients with sporadic CRC. This defect in repairing DNA causes genetic microsatellite instability (MSI+). Patients with MSI+ tumours have a much better prognosis when compared to patients with tumours without this defect.\textsuperscript{56} The reason for this is unclear, but MSI+ tumours are associated with high number of tumour infiltrating lymphocytes, cells that may be protective for the host.\textsuperscript{57}

**p53**

TP53, the well-known tumour suppressor gene, is mutated in up to 50\% of all human cancer.\textsuperscript{58} The p53 protein is acting to induce the cell cycle arrest or programmed cell death (apoptosis) in case of DNA damage, thereby maintaining the genetic stability in the organism. Mutations in the p53 gene generally results in a more stable protein and overexpression of p53 can be detected in IHC.\textsuperscript{59} However, the p53
overexpression can be caused by other mechanisms than mutations. This may be one reason why the prognostic value of p53 in survival analyses in CRC has been limited.\textsuperscript{6} Better methods in identifying specific mutations instead of only accumulation of the p53 protein might improve its prognostic value.\textsuperscript{59,61} Patients with normal p53 genotype have also been shown to respond better to preoperative radiotherapy in rectal cancer, while overexpression of the p53 protein was not related to response or survival.\textsuperscript{62}

**APC**
The adenomatous polyposis coli (APC) gene functions as a tumour-suppressor gene regulating proliferation and cellular adhesion.\textsuperscript{63} Germline mutations of APC causes the familial adenomatous polyposis (FAP) syndrome.\textsuperscript{9} Mutations in APC are also present in 60-80\% of sporadic CRC, representing one of the most common mutations in CRC. They are also present very early during the adenoma formation. APC mutations have no prognostic value in sporadic CRC.\textsuperscript{9}

**Bcl-2**
Bcl-2, originally described in B cell leukemia/lymfoma, is a proto-oncogene, which protein prolongs survival of the cells by blocking apoptosis.\textsuperscript{64} There is disagreement as to the prognostic value of bcl-2 expression in CRC. Some studies have described a better prognosis for patients with bcl-2 immunoreactive tumours\textsuperscript{65,66} while others have come to the opposite conclusion.\textsuperscript{67}

**DCC**
The tumour-suppressor gene called "deleted in colon cancer" (DCC), is deleted in about 70\% of CRC.\textsuperscript{5} The gene, located on chromosome 18q, encodes for a cell surface receptor with homology to the neural cell adhesion molecule (N-CAM) and other related cell surface proteins in the Ig superfamily. Inactivation of the DCC function correlates to decreases in cell-to-cell interaction and attachment, which may increase the potential for metastasis.\textsuperscript{68} Lack of expression of DCC\textsuperscript{69} and presence of 18q LOH\textsuperscript{70} are both negative prognostic factors for survival in stage II and stage III CRC, respectively.

**K-ras**
Ras represent a family of genes (H-ras, N-ras, K-ras) encoding for proteins involved in the transduction of growth and differentiation signals.\textsuperscript{71} K-ras has been extensively studied in CRC, and mutations of K-ras, especially mutation of codon 12, are present in 40-50\% of CRC.\textsuperscript{71} Mutations have in some studies been associated with worsened prognosis.\textsuperscript{72} The presence of mutant ras may also predict poor response to chemotherapy.\textsuperscript{73}

**Thymidylate synthase (TS)**
TS is an enzyme involved in the DNA synthesis and the target for 5-fluoropyrimidine, the most commonly used cytotoxic drug in treatment of CRC.\textsuperscript{74} Detection of TS in tumour tissue has been made using enzymatic, IHC, or RT-PCR methods. High levels of TS have been associated with poor prognosis and resistance to chemotherapy.\textsuperscript{75,76}

**Microvessel density**
The number of blood vessels in CRC tumours, reflecting tumour angiogenesis, has in one multivariate analysis study turned out as an adverse prognostic factor\textsuperscript{77} but there are other reports demonstrating lack of prognostic value\textsuperscript{78} or even increased survival in patients with tumours showing high numbers of blood vessels.\textsuperscript{79}

**Cytokeratins**
Cytokeratins (CK) are the most abundant epithelial protein with the capacity to assemble into filaments. CK are vital for the intracellular skeleton providing mechanical integrity to cells and without them the cells become fragile and prone to rupture. Other emerging functions include roles in cell signaling, the stress response...
and apoptosis. The 20 different polypeptides known today are divided into two families, type I CK (No. 9-20, 40-56.5 kD) and type II CK (no. 1-8, 53-67 kD). The CK form heterodimers made of type I and type II chains in a 1:1 molar ratio. The most abundant epithelial CK are No. 8, 18 and 19, forming the heterodimers 8/18 and 8/19. The CK constitute useful markers in IHC, enabling the discrimination of epithelial cells from mesenchymal or lymphoid cells. Detection using CK as a marker for disseminated disease when positive outside the epithelial compartment have also been used in highly sensitive techniques like RT-PCR. However, the specificity of CK have been limited due to illegitimate transcription, pseudogenes and expressions of CK in normal lymph node, blood and bone marrow.

CK have also been used as tumour markers detecting fragments of degraded CK from malignant cells in serological assays like tissue polypeptide antigen (TPA) and specific TPA antigen (TPS). However, their clinical usefulness in CRC has not been apparent.

Immune cells
The immune system is thought to be of importance in the defence against cancer. A higher number of lymphocytes has been reported in colon cancer than in the normal colon. The lymphocytes are mainly T-lymphocytes and helper-inducer (CD4+), and cytotoxic-suppressor (CD8+) cells, and these cells are particularly located in the stroma. Macrophages (CD68+) are also in abundance in the stroma. Histopathologic studies have reported, that lymphocyte infiltration along the invasive margins of rectal cancers is associated with better survival. However, the clinical use of the classification of Jass et al. has been limited due to lack of standardisation in judging the character of the invasive margin (expanding or infiltrating), and peritumoural lymphocytic infiltration (present or absent). An immune reaction along the invasive margin of CRC with cell-to-cell contact between macrophages (CD68+) and T-lymphocytes (CD4+ or CD8+) as a possible antitumour immunity reaction has been described. However, Cianchi et al. could not detect any survival difference between patients with or without peritumoural lymphocyte infiltration. Othe studies have confirmed the importance of immunological factors. Recently, the number of tumour infiltrating lymphocytes (TILs) has been found to be an independent prognostic factor both for overall survival and disease-free survival in patients with curatively resected CRC and for survival in patients with disseminated disease, respectively. A small portion of these cells are natural killer (NK) cells, and high numbers of infiltrating NK cells is associated with a favorable tumour outcome. The number of CD8+ T-cells infiltrating within human primary CRC cells nests has been shown to be associated with better survival. Changes in immune factors have also been detected in the systemic circulation, like a reduction in circulating CD4+ T-lymphocytes before a recurrence of CRC. Tumour infiltrating lymphocytes have been shown to be more common in tumours having DNA mismatch repair gene mutations resulting in microsatellite instability (MSI). MSI positive tumours have a good prognosis, thus TIL may be a positive prognostic marker for this reason.

Metalloproteinases
Tissue remodelling is essential in normal tissues (eg. in ovulation and wound healing), and in various benign disorders with extensive degradation of extracellular matrix, e.g. rheumatoid arthritis. However, it is also of major importance for tumour progression, invasion, and metastasis. Matrix metalloproteinases (MMPs), which includes at least 23 different enzymes secreted as proenzymes, play a major roll in this degradation of extracellular matrix. These proenzymes, which need to be activated, are also tightly
balanced by four known specific tissue inhibitors of metalloproteinases (TIMPs). In CRC, degradation of the basement membrane is of great importance for invasion. Basement membranes are thin sheetlike structures separating epithelial cells from adjacent stroma. They are important for both differentiation and adhesion, as well as in maintenance and remodelling of organs, like the skin. Native type IV collagen is only present in and also the major constituent of basement membranes, but they also contain other macromolecules as laminin and proteoglycans. Type IV collagen can be degraded by a subgroup of MMPs known as gelatinases A and B (also known as 72kD and 92kD type IV collagenases or as MMP-2 and MMP-9). Matrilysin (MMP-7), is another MMP with broad proteolytic activity, that has been linked to a more aggressive, invasive activity and ability to form metastases. Among the inhibitors, TIMP-3 was recently shown to be a basement membrane-associated protein, with levels progressively decreased with advancing CRC stage.

Of all MMPs, only matrilysin (MMP-7) and membrane type-1 MMP (MT1-MMP) can be secreted by the tumour cell itself, whereas other MMPs, such as MMP-2, is secreted by activated surrounding normal stroma cells (mainly fibroblasts), and MMP-9 predominantly by inflammatory cells. Activation of MMPs cause induction of specific TIMPs from stromal cells to control proteinase reactions. This activation of the surrounding stroma cells can be induced by tumour derived diffusible factors like EGF, TGF-α, TNF-α and IL-1β. In several malignancies, an imbalance in tumour tissue between MMPs and TIMPs has been observed. Recently Baker et al. detected significantly higher levels of active MMP-2, active MMP-9, and also of MMP-1, MMP-2, TIMP-1, and TIMP-2 in tumour tissue compared to normal mucosa. MMP-1 and TIMP-1 levels also correlated significantly with Dukes’ stages.

Ring et al. found that positive expression of TIMP-2 in basement membranes and in stroma correlated to more localised tumours and to longer survival time. However, the difference in survival rate was not valid when only the potentially cured patients were analysed. New techniques using enzyme-linked immunosorbent assays (ELISA) have made it possible to analyse MMPs and TIMPs in blood samples. Elevated levels of MMPs and their inhibitors in blood have been reported in various non-malignant disorders related to enhanced remodelling of tissue such as rheumatoid arthritis, liver disease and glomerulonephritis. In CRC, Zucker et al. showed elevated levels of plasma MMP-9 in patients compared to a control group, but without differences between patients with localized compared to metastatic disease. In another study, they found increased plasma levels of MMP-9 and/or the MMP-9/TIMP-complex in 36% of 63 CRC patients, and a shorter survival in stage IV patients with elevated plasma levels. The results of different studies of MMPs and TIMPs seem sometimes conflicting. This may be the result from small series of patients and/or different techniques. Furthermore, IHC on tissue cannot differentiate between latent or activated forms of MMPs, while substrate gel electrophoresis (zymography) can do so. However, neither technique can be used to distinguish between free MMP and MMP/TIMP complexes. Blood analysis are sometimes done analysing the free (both latent and active) MMPs, the MMP/TIMP complex or total amounts (free and in complex) of MMPs or TIMPs, thus the method of analysis must be considered when comparing different studies.

Micrometastases (MM)
The dissemination of tumour cells via blood or lymphatics, requires invasion into vessels followed by dissemination via
blood or lymphatics. During this phase, isolated tumour cells or cluster of cells may be detected in blood, lymphatic vessels, and lymph sinus of lymph nodes. To form metastases, implantation is needed when tumour cells are arrested in the vessels followed by penetration of the vessel wall and proliferation. This tumour cell proliferation is accompanied by stromal reaction, and for growth beyond 1-2 mm in diameter dependent on angiogenesis. Also in lymph nodes, adhesion, implantation with stromal reaction, and proliferation within the lymphatic tissue is seen. However, no penetration of vessel wall is necessary for cells entering the lymph node through lymphatic vessels. Metastatic tumour in the regional lymph nodes is one of the most important prognostic indicators. These malignant deposits can sometimes be very tiny, although they may be identified using routine hematoxylin/eosin staining, thus the denomination micrometastases (MM) was introduced. MM was originally used for aggregates of tumour cells measuring less then 2 mm in diameter at a distance from the primary tumour. In routine stainings, metastatic single cells or small clusters of tumour cells may be overlooked, but easily identified using IHC methods. The term MM have often been used denoting such isolated tumour cells and also in molecular techniques for positive expression of marker genes in lymph nodes, bone marrow, and peripheral blood causing confusion in terminology. A more appropriate denomination is either "isolated tumour cells", as suggested by UICC or "disseminated or circulating tumour cells". Perioperative findings of isolated tumour cells in the bone marrow or in peritoneal or pleural washings without any macroscopic evidence of residual tumour have resulted in the denomination “minimal residual disease” (MRD). A distinction between true MM, isolated tumour cells, and detection of molecular markers, and between conventional R-classification and MRD are important, since they have different prognostic implications. Therefore a uniform classification including the method of detection is recommended. The MM has a potential for growth and development into life-threatening disseminated disease. The lack of a specific blood supply makes the MM dependent on passive diffusion of nutrients and oxygen, and this may be one reason that limits their growth. The limited growth can be further reduced by apoptosis caused by the immune system, thus the MM seems to be in dormancy. However, if new vessels are created, the possibilities for growth, invasion and distant spread of the malignant cells are enhanced. Angiogenesis is stimulated by hypoxia and numerous cytokines, among other VEGF, and FGF. Some of these stimulating factors for angiogenesis are released in wound healing, and this may be one reason for enhanced tumour growth after surgical procedures. Another interesting phenomenon is the discovery, that the primary tumour can produce inhibitory factors for angiogenesis in metastases, why removal of the primary tumour may result in metastatic progression. Several endogenic anti-angiogenic factors have been identified, like angiostatin and endostatin. This kind of factors may suggest a new strategy when treating malignancies. The clinical implications of MM and MRD have been evaluated in numerous studies with disparate results and the prognostic significance of these tumour cells or detected gene material awaits further affirmation. Most studies have not evaluated true MM, but isolated or disseminated tumour cells or findings in molecular methods, where metastatic phases like implantation, proliferation, and stromal reactions cannot be seen. Larger prospective studies with standardised techniques and sufficient follow-up are needed. Only then can the prognostic significance of MM be evaluated. This is
important to evaluate before they are incorporated into staging classification, thereby avoiding stage migration, which would make it hard to compare new treatments with historic data. The positive effect of standard adjuvant chemotherapy also has to be verified in MRD since the dormant cells may be less sensitive to chemotherapy. Immune therapy, using monoclonal antibodies against the 17-1A antigen (Panorex; Glaxo Wellcome, Hamburg, Germany) has in one study shown a substantial reduction in mortality, however, a recent phase III study using Edrecolomab also directed against the 17-1A antigen could not verify this.

Detection methods of disseminated disease
Handling of specimen and lymph nodes
The resected specimens should be labelled carefully with suture-markings, making the orientation of the specimen and the identifications of central ligatures of vessels easier for the pathologist. The histopathologic examination of the resected specimen is of highest importance for securing an adequate staging of the tumours. The primary tumour is carefully dissected fresh or after formalin fixation to secure sufficient samples for the examinations. Radial margins should be marked with ink. Lymph nodes should be collected and marked if located along main vascular trunks. The number of nodes examined, necessary to avoid understaging has been subject to intensive discussion and the current number advocated is at least 12 lymph nodes. Fat-clearance methods have been used increasing the number of harvested lymph nodes, but some authors suggest adequate fixation of the mesenteric fat prior to dissection.

Sentinel node and radioimmunoguided surgery
The sentinel node technique was introduced to identify the primary tumour draining lymph nodes being at highest risk for metastases. Dye and/or radiolabelled colloid was injected in vivo at the site of the primary tumour or dye injected ex vivo into the specimen. The high risk lymph nodes can then be accurately examined and the total work-load may be reduced. The frequency of false negative sentinel nodes, eg. skip lymph nodes, are, however, a major concern ranging from 0-60% in different studies, thus the method needs to be evaluated in larger prospective trials. The use of this technique in breast cancer can reduce morbidity, if the axillary lymph node dissection safely can be omitted on patients with negative sentinel nodes. However, in CRC wide radical lymph node resection is advocated, and the resection lines are also determined by vascularisation of the intestines offering safe conditions for healing of anastomosis. In exceptional cases abnormal lymph drainage can be detected with sentinel nodes outside the intended specimen, which may alter and extend the performed surgical procedure.

Radioimmunoguided surgery (RIGS), using a radiolabelled antibody towards tumour antigens injected prior to surgery with preoperative scintigraphy and/or intraoperative detection using a hand-held gamma-detecting probe, has also been tested for identification of metastatic disease. Remaining radioactivity at the end of surgery resulted in bad prognosis even after excision of all hematoxylin/eosin positive lesions.

Microscopic examination
The microscopic evaluation is still most often done on slides routinely stained with hematoxylin/eosin. Examinations of multiple sections at different levels of the lymph node improves detection of metastatic disease. IHC stainings using monoclonal antibodies (often anti-cytokeratin ab), detecting cells of epithelial origin, is essential when looking for single, malignant cells, but can also make the diagnoses of multiple metastatic cells in lymph nodes.
A precise cytologic interpretation of the stained cells is also mandatory to exclude false positive cells.140

Molecular techniques
Modern highly sensitive molecular methods have been developed using the polymerase chain reaction (PCR) method.119 Using PCR, a selected specific DNA sequence can be amplified, doubling the number of the selected DNA fragments for every repetitive series of cycles. An amplification using 30 PCR cycles results in amplification around 1000 million times. PCR can also be used to amplify RNA, but then the reaction starts using the enzyme reverse transcriptase (RT) for reverse transcription of RNA into cDNA and the method is called RT-PCR.119

Another improvement is the development of quantitative RT-PCR which makes it possible to not only identify the marker, but also quantify it using standard curves with known amounts of mRNA for the marker.142

A main problem in using these molecular techniques is the lack of reliable markers. In PCR, a tumour-specific mutation in DNA must be selected for identification of disseminating tumour cells. However, there is a vast heterogeneity in tumours, and there is no known mutation occurring consistently in CRC.

Hayashi el al.143 studied a PCR–based assay, mutant-allele-specific amplification (MASA). They first detected tumour-specific DNA mutations (p53 and K-ras) in the primary tumours before detecting the same mutations in the lymph nodes. Relapses in CRC were much more common among the patients who had the same mutation in p53 or K-ras in their lymph nodes than patients without mutations (27 relapses in 37 patients vs 0 relapse in 34 patients). However, in 41% of the patients no suitable mutation in the primary tumour could be found.

The RT-PCR allows the identification of disseminated tumour cells by detecting tissue-specific mRNA like cytokeratins and CEA. These proteins are not tumour specific but are selectively expressed in epithelial cells and consequently indicators of tumour cells when detected outside epithelial compartments.40,80 The major problem has been to find a sufficiently specific marker.81 The cytokeratins CK8, 18, and 19 have all been shown in illegitimate transcription (transcription of any gene in any cell type) in bone marrow, and to be expressed in normal lymph nodes and polymorphonuclear blood cells. Furthermore, pseudogenes have been described. They are non-functional DNA lacking an intronic sequence and when subjected to RT-PCR result in a product identical to that of mRNA amplification. CK20 has been found in normal blood and granulocytes.81,120

Most studied of all tumour markers is the CEA. Positive expression of CEA mRNA has in some studies been found in high percentage in peripheral blood, bone marrow, and lymph nodes among healthy controls.81 However, the pronounced homology between different members in the large CEA family makes crossreaction common and must be considered when selecting the marker sequence in mRNA.

Another problem is contamination from skin cells in venipuncture, shedding of non-viable tumour cells or tumour cells debris into blood, bone marrow, and lymph nodes, all resulting in false-positive results.145

A further obstacle is the instability of mRNA, in contrast to DNA, which has demanded detection in fresh or fresh-frozen materials. New improvements make it now possible to use also archival paraffin embedded material.146 A technique analysing single cells captured after laser microbeam microdissection and laser pressure catapulting has also been developed.147,148

Recently, a new simple method of protein extraction and determination of CEA concentrations in lymph nodes for
detection of lymph node metastases was presented.\textsuperscript{149}

**Detection in other compartments**

In order to detect malignant cells in bone marrow as well as in peripheral or tumours draining portal blood, ICC, flow cytometry and RT-PCR can be performed.\textsuperscript{81,114} A way to obtain a sample enriched of tumour cells is density-gradient centrifugation enrichment (often using Ficoll-solutions), where most tumours cells are found in the mononuclear cell fraction separated from peripheral blood according to cell density.\textsuperscript{150,151}

The density-gradient enrichment can be combined with the use of immunomagnetic beads to obtain a sample further enriched in tumour cells and reducing the background of normal cells.\textsuperscript{119,152} In this technique, the sample is incubated with magnetic beads coated with antibodies against epithelial cells and the separation done using a powerful magnet. Enrichment using magnetic beads isolating mRNA is also possible, which may increase the sensitivity and specificity of the RT-PCR.\textsuperscript{119} Detection of exfoliated cancer cells in the peritoneal cavity has been achieved mostly using peritoneal lavage\textsuperscript{35,153} or by pressing glass histology slides over the surface of the fresh specimen.\textsuperscript{154} Processing of the lavage can incorporate the same techniques as for blood, using density-gradient enrichment, magnetic beads and ICC. Also detection using quantitative RT-PCR has been done.\textsuperscript{38}
AIMS

The overall purpose of this thesis was to study methods to improve the selection of patients for adjuvant chemotherapy. The specific aims can be outlined as follows:

- To assess the impact on prognosis of disseminated malignant cells in regional lymph nodes in Dukes’ stages A and B CRC.

- To evaluate the prognostic value of preoperative serum levels of metalloproteinases MMP-2 and MMP-9 and their inhibitors TIMP-1 and TIMP-2 for staging and prognosis in CRC.

- To study the prognostic impact of the number of immune cells in regional lymph node metastases in Dukes’ stage C CRC.

- To examine the possibilities of using quantitative RT-PCR in detecting CEA mRNA as a marker for disseminated tumour cells in lymph nodes.
MATERIALS & METHODS

Patients
The patients enrolled in the four papers underwent surgery at the Department of Surgery, University Hospital, Umeå, Sweden, except for 66 patients in paper II that had their surgery done at four nearby county hospitals. The studies were performed in a retrospective way in paper I-III, and the samples were collected prospectively in paper IV. All studies were done with approval from the local ethical committee.

In paper I, 147 consecutive patients with CRC in Dukes’ stages A or B operated on between 1987 and 1994 were studied. Of these, 24 patients were also included in paper II.

In paper II, 158 patients with tumours in Dukes’ stages A-D, operated on between 1992 and 1996, were analysed. As controls 80 healthy blood donors were selected.

In paper III, 93 patients with tumours in Dukes’ stage C treated from 1987 to 1996 were studied, 16 patients were also included in paper II.

In paper IV, 51 patients Dukes’ stages A-D operated on between 1998 and 2001 were studied. As controls, 10 patients operated on for a non-malignant colorectal disease were used.

The patients are further described in each paper.

Samples
In papers I and III, archival formalin-fixed, paraffin-embedded materials were retrieved for analyses. In paper IV lymph nodes were dissected from the fresh specimens. The nodes were cut into halves with separate knives under sterile conditions to prevent RNA cross-contamination between specimens. One half of each node was fixed in 10% buffered formalin for routine histopathology and IHC, the other half was stored at -70°C until mRNA extraction.

In papers II and IV, blood sampling was done on the morning prior to or on the same morning as the surgical procedure. After coagulation the serum was separated by centrifugation (1500g for 15 min), and then stored at -70°C. In the control group of paper II, blood sampling, processing, and storage were as described above. All blood samples were simultaneously analysed for each factor.

Immunohistochemistry
A single 4-µm section from each lymph node was stained in papers I, III and IV for each antibody, respectively. An automatic IHC machine, VentanaTM 320-202 (Ventana Medical Systems, Inc., Tucson, AZ) was used for the cytokeratin staining in paper I.

An antigen retrieval procedure followed by the ABC method was applied using monoclonal mouse antibodies and the Vectastain ABC Elie kit (Vector Laboratories, Burlingame, CA) in papers III and IV. The antibodies used were all mouse monoclonal antibodies. Table 2.

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Table 2. Monoclonal antibodies used in the studies.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Main reactivity</th>
<th>Clone</th>
<th>Distributor</th>
<th>Working dilution</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokeratin</td>
<td>cytokeratin 8 and 18</td>
<td>CAM 5.2</td>
<td>Becton Dickinson, Mountain View, CA</td>
<td>1:100</td>
<td>I</td>
</tr>
<tr>
<td>CEA</td>
<td>carcinoembryonic antigen</td>
<td>II-7</td>
<td>Dako A/S, Glostrup, Denmark</td>
<td>1:40</td>
<td>IV</td>
</tr>
<tr>
<td>CD8</td>
<td>cytotoxic / suppressor Tcells</td>
<td>8/144B</td>
<td>Dako A/S, Glostrup, Denmark</td>
<td>1:40</td>
<td>III</td>
</tr>
<tr>
<td>CD45R0</td>
<td>activated immune cells and memory Tcells</td>
<td>OPD-4</td>
<td>Dako A/S, Glostrup, Denmark</td>
<td>1:60</td>
<td>III</td>
</tr>
<tr>
<td>CD68</td>
<td>tissue macrophages and cytotoxic lymphocytes</td>
<td>PG-M1</td>
<td>Dako A/S, Glostrup, Denmark</td>
<td>1:60</td>
<td>III</td>
</tr>
</tbody>
</table>
Histopathology
Sections of formalin-fixed, paraffin embedded primary tumours and lymph node tissue were stained with hematoxylin/eosin and subjected to routine histopathological evaluation at the Department of Pathology, Umeå University Hospital.

Microscopic Evaluation
Each section was examined simultaneously by two of the investigators. Consensus was reached for the assessment of each section. The occurrence of one or more cytokeratin-positive cell (paper I), or CEA-positive cell (paper IV), in at least one lymph node section from the resected specimens was indicative for the occurrence of "micrometastases". The microscopic evaluation included a cytologic interpretation in order to exclude false positive cells and cell debris.

In paper III, the cancer cell stroma, which displayed the highest number of positive cells in the lymph node metastases was evaluated. The number of immune cells in the lymph nodes were categorised as either none or few (<5), sparse (5-15), or many (>15) positive cells per field of vision at 500 magnification.

Detection of metalloproteinases
ELISAs were performed on 96-well microtiter plates (Nunc Maxisorb, Denmark) using standard protocols. A polyclonal antibody, produced from chicken against each of the analytes, was used as secondary antibody. A peroxidase labelled anti-chicken-IgG (Chemicon, Pittsburgh, PA) was used for detection of the bound secondary antibody. OPD-tablets (KemEnTec, Denmark) were used to visualize the peroxidase label. The colour formation was measured on 450 nm (Anthos 2000 microplate reader, Anthos, Salzburg, Austria), and calculations were done using a Multicalc program (Wallac, Finland). All standard curves were done with purified recombinant proteins and the concentrations were calculated by measuring the absorbance. The ELISA protocol is shown in Figure 4 and the precision data for MMP/TIMP ELISA kits are shown in paper II.

For quantitation of MMP-2, two different ELISAs were used: one measuring the free form, i.e. not bound to its inhibitor, and the other measuring the MMP-2/TIMP-2 complex. For the detection of MMP-9, TIMP-1, and TIMP-2, the total protein was measured, i.e. a protocol to simultaneously quantitate the free and complex forms was used.

Figure 4. Principle of the ELISA method. In panel A the detection of free MMP by coating the plate with TIMP onto which the MMP in the sample binds and is then visualised through a polyclonal anti-MMP. In panel B the detection of MMP/TIMP complex by coating the plate with anti-TIMP monoclonal antibody and the bound complex is visualised through a polyclonal anti-MMP antibody. In panel C the detection of total TIMP by coating the plate with a monoclonal anti-TIMP antibody which detects both the free TIMP and MMP/TIMP complex. The visualisation is accomplished with a polyclonal anti-TIMP antibody. In panel D the detection of total MMP is shown with the same principle as with total TIMP in panel C, but using anti-MMP antibodies instead.
Determination of CEA concentration in serum

Samples were analysed in duplicate for CEA concentration by ELISA according to the protocol of the manufacturer (ICN Biomedical Research Products, Costa Mesa, CA). All samples were analysed on the same occasion.

Blood cells

Peripheral blood mononuclear cells (PBMC) and granulocytes were isolated from heparinized peripheral blood or buffy coats of healthy adults by Ficoll-Isopaque gradient centrifugation as described. In vitro activated PBMC were obtained by incubation in HEPES buffered RPMI1640 supplemented with 0.4% human serum albumin with addition of either 50 ng/ml of the anti-CD3 mAb OKT3, 3 µg/ml of the T cell mitogen Concanavalin A or a combination of phorbolmyristateacetate (5 µg/ml) and Ionomycin (1µg/ml) as activators. PBMC were incubated with these stimulants in parallel cultures for 4, 7, 20, 48 and 72 hours, washed, snap frozen and stored at -70°C until RNA extraction.

Colorectal cancer tissue

Immediately after resection of the tumour specimens, an approximately 0.5 x 0.5 x 0.5 cm piece was collected from the outer rim, snap-frozen, and kept at -70°C until RNA extraction.

Tumour cell lines

The well-differentiated human colon cancer cell line LS174T, the moderately differentiated human colon cancer cell line HT-29, and the human promyelocytic leukemia cell line HL-60 were grown in Parker199 medium (SBL Vaccin, Stockholm, Sweden) supplemented with 8% fetal calf serum and antibiotics.

RNA preparation and real-time quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from tissues and cells by the acid guanidine-phenol-chloroform method. Precipitated RNA was dissolved in RNase free water containing 1000 U/ml of the ribonuclease inhibitor rRNasin (Promega, Madison, WI) and stored at -70°C. Total amount of RNA was determined by OD260 measurements in three PBMC samples and five lymph node samples prior to the addition of rRNasin. Purity was ascertained by an OD260/OD280 ratio >1.8.

A real-time qRT-PCR assay for quantitative determination of CEA mRNA was constructed using the TaqMan EZ™ technology (Applied Biosystems Division, Perkin-Elmer, Foster City, CA). Specific primer pairs placed in different exons were used together with a reporter dye-labelled probe hybridizing over two UTR-exons with an intervening intron in the amplicon.

Figure 5. Sequences were 5'-CTGATATA GCAGCCTGGTGAGT-3', 5'-TGTGG CAAATGCTTTAAGGAAGA-3' and 5'- TTATTTCAGGAAGACTGACAGTTTGCT-3' for the 5'-primer, the 3'-primer and the probe, respectively. Emission from released reporter dye was monitored by the ABI Prism 7700 Sequence Detection System (Applied Biosystems Division). The RT-PCR profile was: 50°C for 2 min, 60°C for 30 min, 95°C for 5 min followed by 45-50 cycles of 94°C 20 s and 60-62°C for 1 min. Total RNA extracted from LS174T cells was used for optimisation of the assay. The assay gave a linear relation between log concentrations of standard RNA and PCR cycles over a range of 7 logs. A specific RNA copy standard was prepared for the optimized assay (see below). Analyses were carried out in triplicates and expressed as copies of mRNA/µl as determined from parallel RT-PCR of serial dilutions of the RNA copy standard. The concentration of 18S rRNA was determined for each sample by qRT-PCR according to the instructions of the manufacturer (Applied Biosystems Division). No copy standard is available for the 18S rRNA assay. Therefore, the 18S rRNA content was expressed as arbitrary units defined as the amount of 18S rRNA in 1 pg total RNA.
Figure 5. Schematic view of the process of real-time qPCR. After the mRNA is reversed transcribed to cDNA the primers and the probe bind to the polymerisation step. During strand displacement the polymerase breaks the bond between the probe and the DNA strand and a new DNA strand is forming. The probe is then cleaved and the fluorochrome released and the fluorescence measured.

Figure 6. Section from a regional lymph node showing many CD45R0+ cells. Original magnification x500.
extracted from PBMC. Results are expressed as mRNA copies of CEA mRNA per unit of 18S rRNA. Quantitative PCR assays for determination of possible DNA signals were performed using the same primers, probe and PCR profile without RT step and using AmpliTagGOLD DNA polymerase (Applied Biosystems Division) instead of recombinant thermostable *Thermus thermophilus* (rTth) polymerase. All samples of RNA extracted from lymph nodes were checked for presence of immune cell RNA by RT-PCR for CD45 in order to certify the correct tissue origin of the RNA (see Lundqvist et al.160 for primer sequences).

**Cloning and sequencing**

RT-PCR products were subjected to gel electrophoresis in 1.5-2% SeaKem®LM agarose (FMC BioProducts, Rockland, ME), fragments were cut out and purified with QiaexII® Gel extraction kit (Qiagen, Hilden, Germany) and then ligated into dT-treated EcoRV pBluescript (SK+ or KS+). Plasmids were transformed into competent *E. coli* XL1blueMRF’ and grown on plates containing 100 µg/ml ampicillin, 12.5 µg/ml tetracycline, 20 µg/ml X-gal, and 40 µg/ml IPTG. Transformants were checked for insert of the expected size on agarose gel after restriction cleavage of plasmids using Xbal/XhoI (Life Technologies, Gaithersburg, MD). Two to three µg DNA was used for cycle-sequencing using T7 or Rev primers and Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Piscataway, NJ). Cycle-sequenced products were analyzed by ALFexpress (Amersham Pharmacia Biotech).

**RNA copy standard preparation**

Total RNA from the colon adenocarcinoma cell line LS174T was used as starting material for copy standard preparation. RT-PCR was performed using specific 3′-primers in combination with rTth polymerase for reverse transcription and primers placed flanking the amplicon in the qRT-PCR assay for PCR. The sequences were 5′-CTGATATAGCAGCCCTGTTG TAGT-3′ and 5′-TGTTGCAAATGCTTTAGGAAGA-3′ for the 5′-primer and the 3′-primer, respectively. The RT-PCR product was cloned, sequenced and used as template for *in vitro* transcription with T7 polymerase/ RiboProbe® In Vitro Transcription Systems (Promega) or T7-MEGAshortscript™ (Ambion). A 7 µg linearized DNA, or 0.5 µg for T7-MEGAshortscript™, was used in a large scale synthesis reaction carried out at 37°C for 2-3 h. The reaction products were then treated with 1U/µg of RNase-free DNase (Promega) for 30-40 min at 37°C, followed by extractions with phenol: chloroform: isoamylalcohol (25:24:1) and chloroform: isoamylalcohol (24:1). RNA was precipitated with 2.5 volumes of 99.5% ethanol and 0.5 volumes of 7.5 M ammonium acetate at -70°C for at least 1 h. DNase treatment was repeated at least twice. Finally, the copy standards were checked by RT-PCR and PCR to evaluate the content of DNA which proved to be less than 0.1%. Concentration of the transcripts was calculated on the basis of the OD260 value, the molecular weight of the transcript and Avogadros’ number. The standard was finally diluted to 10⁸ copies/µl.

**Statistics**

Statistical calculations in papers I-III were performed using the statistical software SPSS (version 6.1.3, SPSS Inc, Chicago, IL), and in paper IV the statistical software GraphPad Prism (version 2.0, GraphPad Software Inc, San Diego, CA).

In papers I and IV the Pearson’s chi-squared test was used to test differences in distribution between groups. In papers II and III the Mann-Whitney U and Kruskal-Wallis 1-way Anova with independent samples were used to test differences between groups. In paper IV the Mann-Whitney’s rank sum test was used testing differences between patient groups and Wilcoxon’s assigned test was used...
comparing values from the same individual. The significance of differences in frequencies between patient groups was determined by Fisher's exact test. In all papers Spearman's correlation coefficient was used to test for correlation. Survival curves were constructed by the Kaplan-Meier method, and differences between curves were tested by log-rank test. A P-value <0.05 was considered to indicate a statistically significant difference.
RESULTS

Paper I

The microscopic evaluation revealed cytokeratin-positive cells, most often as single cells, and sometimes as clusters of cells. (See cover picture) The cells were observed mainly in the peripheral sinuses of the lymph nodes. There were a total of 77 positive lymph nodes out of 609 analysed (11.6%). A total of 47 out of 147 patients (32%) had positive cells with no difference between Dukes’ stages A and B (34% vs. 31%) However, patients with rectal cancers showed more often cytokeratin positive cells compared to colon cancer patients (47% vs. 24%; P=0.004). At follow-up in June 1996, a total of 23 patients had died from cancer and another 28 from non-related disease, resulting in a median follow-up of the 96 living patients of 54 (range 18-111) months. The cancer-related five-year survival rate for patients with tumours in Dukes’ stage A was 92% and in stage B 72% (log-rank test, P=0.042). There were 8 cancer-related deaths in 47 patients (17%) with lymph nodes with cytokeratin-positive cells, and 15 in 100 patients (15%) with negative lymph nodes. In the survival analysis, no differences in cancer specific survival time were observed between patients with or without cytokeratin-positive cells in their regional lymph nodes, irrespective if all patients or patients in the separated Dukes’ stages A or B were studied. Also, there was no significant difference in the survival rate between patients with tumours in the colon or in the rectum.

In a repeated follow-up in June 2001, another 9 patients had died from CRC, including all six patients with known tumour relapse in the first follow-up. A total of 47 patients had died of other causes than cancer, leaving 68 living patients with a median follow-up time of 109 (79-172) months. The cancer-related 10 years survival rate for patients with tumours in Dukes’ stage A was 84% and in stage B 69% (log-rank test, P=0.046). There were 12 cancer-related deaths in 47 patients (25%) with cytokeratin-positive cells in their lymph nodes and 20 in 100 patients (20%) with negative nodes. Table 3

In the survival analyses, no significant differences could be found in survival time for all patients or between patients operated for Dukes’ A or B tumours respectively in relation to if there were cytokeratin-positive cells in the lymph nodes or not. Figures 7a and 7b.

<table>
<thead>
<tr>
<th>Location/Dukes' stage</th>
<th>Total No.</th>
<th>No. patients with CK positive cells</th>
<th>Cancer Deaths, no. (%)</th>
<th>No. patients without CK positive cells</th>
<th>Cancer Death, no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>23</td>
<td>6</td>
<td>1 (17)</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>73</td>
<td>17</td>
<td>4 (24)</td>
<td>56</td>
<td>13 (23)</td>
</tr>
<tr>
<td>Rectum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>21</td>
<td>9</td>
<td>2 (22)</td>
<td>12</td>
<td>2 (17)</td>
</tr>
<tr>
<td>B</td>
<td>30</td>
<td>15</td>
<td>5 (33)</td>
<td>15</td>
<td>5 (33)</td>
</tr>
<tr>
<td>Total</td>
<td>147</td>
<td>47</td>
<td>12 (25)</td>
<td>100</td>
<td>20 (20)</td>
</tr>
</tbody>
</table>
**Figure 7a.** Survival curves for patients in Dukes’ stage A. Patients with (solid line) or without (dotted line) cytokeratin positive cells in their lymph nodes.

**Figure 7b.** Survival curves for patients in Dukes’ stage B. Patients with (solid line) or without (dotted line) cytokeratin positive cells in their lymph nodes.
The values for all serum levels in controls and in patients are given as Whisker-and-Box plots in Figure 8. Healthy men, compared to healthy women had significantly higher levels of MMP-9 (mean 62ng/ml vs. 41ng/ml, P<0.01), and TIMP-2 (mean 165ng/ml vs. 156ng/ml, P<0.05). No significant differences according to gender were observed for the other factors analysed. A positive correlation to age was noticed for TIMP-1 (P<0.001) and for MMP-2/TIMP-2 complex (P<0.05), whereas this was not the case for the other factors.

Using the reference limits (mean+2SD) set from analyses in the control group, the rates for all patients with elevated levels of MMP-2, MMP-9, TIMP-1, and TIMP-2 were 5%, 41%, 54%, and 66%, respectively, whereas 40% had decreased levels of the MMP-2/TIMP-2 complex.

After exclusion of Dukes’ D patients, the corresponding values were 5%, 41%, 47%, 65%, and 37%. Patients with CRC had compared to the control group significantly higher levels of free MMP-2 (P<0.05), MMP-9 (P<0.001), TIMP-1 (P<0.001), and TIMP-2 (P<0.001). The level of the MMP-2/TIMP-2 complex was lower in patients compared to the control group (P<0.001). These results were valid irrespective of if the analyses were performed in all patients or in patients that had a potentially curative resection.

Patients with tumours in Dukes’ stage D had significantly higher levels of TIMP-1 (P<0.001) compared with the potentially curatively resected patients. Figure 8. There were no significant differences between serum levels of free MMP-2, MMP-2/TIMP-2 complex, MMP-9, or TIMP-2 with respect to Dukes’ stage.

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When all patients were included in the survival analysis using the median cut-off value and stratifying for Dukes’ stages, significantly worse prognosis was recorded only for patients with elevated levels of TIMP-2 (log-rank test, \( P=0.027 \)) Figure 9a. However, when the analyses were restricted to the curatively resected patients, there was no significant relation to prognosis for any of the studied MMPs or TIMPs. When these analyses were repeated using the third quartile cut-off value (Q3), only high free MMP-2 correlated to poor survival (\( P<0.05 \)). Figure 9b.

**Figure 9a.** Survival curves for all patients with TIMP-2 levels above (dotted line) or below (solid line) the median cut-off level.

**Figure 9b.** Survival curves for patients with a potentially curative resection (Dukes’ stages A-C) with the MMP-2 level above (dotted line) or below (solid line) the third quartile (Q3) cut-off level.
Paper III

CD8
A majority of the patients (73/90, 81%) had regional lymph node metastases with no or few infiltrating cells with a cytotoxic/suppressor phenotype, CD8+ cells. When this group of patients was compared to the patients with more than 5 cells/field of vision, there was a significantly better prognosis for patients with the higher number of CD8+ immune cells. The cancer specific 5-year survival rate was 77% versus 38% (P=0.011). Figure 10a.

CD45R0
Most patients (54/90, 60%) had regional lymph node metastases with no or few infiltrating immune cells with an activated phenotype, CD45R0+ cells. Similar the CD8 analyses, there was a significantly better prognosis for patients with the higher number of CD45R0+ cells. Figure 6. The cancer specific 5-year survival rate was 66% versus 33% (P=0.002). Figure 10b.

CD68
Many patients (59/91, 65%) had regional lymph node metastases with a sparse number of infiltrating macrophages, CD68+ cells. When this group of patients (including 4 patients with no or few cells) was compared to the patients with many infiltrating cells, there was a significantly better prognosis for patients with many immune cells. The cancer specific 5-year survival rate was 60% versus 37% (P=0.033). Figure 10c.

Correlation and combinations of the three various CD stainings
There was a positive correlation between the number of CD45R0+ and CD8+ immune cells (P<0.001, k=0.53), and between the number of CD45R0+ and CD68+ cells (P<0.001, k=0.41). In contrast, no correlation was observed when comparing the number of CD8+ and CD68+ cells. Suggesting that at least some of the activated cells are cytotoxic T cells.
Survival analyses based on combinations of two or all three CD stainings were all highly significant. However, using various combinations of the three stainings did not increase their individual value as potential prognostic tools. All the analyses resulted in a low risk group with a 5-year survival rate of 60-70%, and a high risk group with a 5-year survival rate of 30-40% (survival curves not shown).

Another statistical analysis was made with exclusion of a total of 19 patients (11 patients, who received preoperative radiotherapy, 7 patients, who received postoperative adjuvant chemotherapy, and 1 patient, who was given both treatments). Similar significant differences as in the total group of CRC patients in the survival rate were observed between patients with high numbers of CD8+ or CD45R0+ immune cells compared to patients with low number of cells, respectively. However, when the same analysis was performed regarding CD68+ cells, the earlier observed difference was no longer retained (P=0.074).
Survival curves for patients with higher numbers of infiltrating CD8+ immune cells (solid line) and those with no or few cells (dotted line).

**Figure 10a.**

Survival curves for patients with higher numbers of infiltrating CD45R0+ immune cells (solid line) and those with no or few cells (dotted line).

**Figure 10b.**

Survival curves for patients with higher numbers of infiltrating CD68+ immune cells (solid line) and those with no, few or sparse cells (dotted line).

**Figure 10c.**
Characteristics of the assay

Primers and probe were placed at and around the M/3' and 3'UTR exon boundary in the CEA mRNA sequence. This region was chosen because it shows low sequence homology with mRNA sequences of all other CEA gene family members. The assay was highly selective for CEA. No signal was obtained in RNA extracted from purified granulocytes or the promyelocytic cell line HL-60, known to express the closely related CEA gene family members BGP, NCA, CGM1 and CGM6. Furthermore, RNA extracted from PBMC of 13 healthy adults showed no signal in nine and a extremely low level in four. Pooled RNA from polyclonally activated PBMC contained no detectable CEA mRNA. A very weak signal was seen when purified DNA was used as template. However, no DNA signal was seen in RNA extracted from any of the lymph nodes in this study (n = 132).

The assay was highly sensitive with a detection limit of 20 copies of CEA mRNA. There was a linear relation between log concentration of standard RNA and PCR cycles up to $10^8$ copies. To mimic the RNA of lymph nodes with disseminated tumour cells we added RNA copy standard diluted in buffer containing total RNA (200 ng/µl) from PBMC for the standard curve. The concentration of added RNA was chosen from the concentration of RNA in lymph node extracts (n = 5) as determined by OD$_{260}$. Figures 11a and 11b show a typical experiment. The reproducibility of the assay was high. RNA from 12 lymph nodes were analysed in 6 independent experiments with new dilutions of the copy standard in each experiment. The variation between experiments was very low at concentrations above 100 copies/µl. Figure 11c. To be able to compare results from different lymph nodes the content of CEA mRNA was normalised to the content of 18S rRNA in the sample. The content of 18S rRNA is likely to differ between the large and active tumour cells/epithelial cells and the smaller metabolically less active immune cells. To estimate what a given CEA mRNA copy/18S rRNA value represents in terms of proportion of tumour cells among the immune cells in the lymph node we performed experiments in which LS174T cells were added to PBMC at different ratios whereafter RNA was extracted and CEA mRNA and 18S rRNA levels determined. There was an almost linear relationship between CEA mRNA copies/18S rRNA unit and tumour cell:PBMC ratio from 1:10000 up to 1:1. One CEA mRNA copy/18S rRNA unit corresponded to approximately 2 LS174T cells per 1000 PBMC. Figure 11d. The influence of PBMC on the CEA mRNA/18S rRNA ratio was only detectable at tumour cell:PBMC ratios below 1:1. Figure 11d. The same type of experiment with HT29 cells gave similar results but with approximately 20 times less CEA mRNA copies per tumour cell (data not shown).

Figure 11a Real-time qRT-PCR results from serial dilutions of CEA RNA copy standard in 200 ng total RNA extracted from PBMC. Values indicate concentration of CEA RNA copies. Each concentration was analyzed in triplicate and the individual curves are shown.
Figure 11b Standard curve from (a) plotted as number of PCR cycles required to reach the threshold above background versus concentration of copies/µl (black dots). Gray dots indicate unknown.

Figure 11c Results from 6 independent determinations of CEA RNA copy concentration in extracts of 12 individual lymph nodes. Each run was performed with parallel external standard as shown in 11a-b.

Figure 11d Results from two independent experiments in which colon carcinoma cells LS174T were admixed with PBMC, RNA extracted from the mixture and the concentrations of CEA mRNA copies and 18S rRNA in the extract determined by real-time qRT-PCR.
CEA mRNA in lymph nodes

One to four regional lymph nodes from 51 CRC patients and 10 control patients were analysed for CEA mRNA. The results are shown in Figure 12a. CEA mRNA levels were below 0.01 copies/18S rRNA unit in all lymph nodes of controls. In contrast, all but one lymph node from patients with cancer in the Dukes’ stage C had CEA mRNA levels above 0.01 copies/18S rRNA unit. Figure 12a. A wide variation in values was seen within each Dukes’ stage, especially in Dukes’ stage B. This was partly due to the fact that individual nodes from the same patient could show highly different CEA mRNA levels. Figure 12b. There was a large variation from almost identical values to differences of several orders of magnitude between individual nodes from the same patient.

We argued, that since the detection of a single lymph node with overt metastasis is sufficient for classification of a tumour as Dukes’ stage C, it is correct to use the lymph node with the highest CEA mRNA level as the indicator for tumour cell infiltration. In Figure 12c only the lymph node with the highest CEA mRNA content for each patient is shown. All control patients had CEA mRNA levels below 3 x 10⁻³ copies/18S rRNA unit. Conversely, all CRC patients, except for 3 in Dukes’ stage B and 2 in stage D, had CEA mRNA levels above 3 x 10⁻³ copies/18S rRNA unit. Figure 12c. On average, patients belonging to all four Dukes’ stages of CRC had significantly higher CEA mRNA levels in lymph nodes than the levels observed in lymph nodes of controls with median CEA mRNA copies/18S rRNA values of 0.02, 0.04, 39.5, and 0.08 for patients with tumours in Dukes’ stage A, B, C, and D, respectively. Comparisons between different Dukes’ stages showed that patients with tumours classified as Dukes’ stage C had significantly higher CEA mRNA levels in their lymph nodes than patients with tumours in Dukes’ stage A or B (P=0.005 and 0.0002 for Dukes’ C compared to Dukes’ A and B, respectively).

The large spread in CEA mRNA expression levels in lymph nodes does not seem to be due to variation in CEA mRNA expression levels in the cells of the original tumour. As can be seen from Figure 12d the CEA mRNA levels varied between 10 and 250 CEA mRNA copies/18S rRNA in RNA extracted from the original tumour of 10 Dukes’ stage B cancer patients and there was no correlation between the CEA mRNA levels in the lymph nodes and the original tumour (P>0.05). Thus, the variation in CEA mRNA expression level is likely to mainly reflect different degree of tumour cell infiltration. Two of the patients with tumours classified as Dukes’ stage B died from CRC. Figure 12c. One had a CEA mRNA level of 3 copies/18S rRNA unit in the highest lymph node, i. e. similar to lymph nodes with overt metastases detected by routine histopathologic examination. The other patient had 0.2 copies/18S rRNA unit, which is within the range of lymph nodes of Dukes’ stage C patients (>0.03 CEA mRNA copies/18S rRNA unit).

There was a large variation in CEA mRNA levels in lymph nodes from patients with cancer in Dukes’ stage D. Figure 12c. Interestingly, the primary tumour of Dukes’s stage D patients with values above 100 CEA mRNA copies/18S rRNA unit were classified as Dukes’ stage C, while primary tumours of those with lower values were classified as Dukes’ stage A and B. Seven patients with tumours classified as Dukes’ stage A, B or C received preoperative irradiation. The CEA mRNA content in their lymph nodes with highest level ranged from 4 x 10⁻³ to 3 copies/18S rRNA unit. Figure 12c. Thus, in this limited material we find no effect of preoperative irradiation on CEA mRNA levels in regional lymph nodes.
Figure 12a CEA mRNA expression levels in lymph nodes collected from specimens removed by colorectal surgery from non-cancer patient (ctr), and cancer patients with tumors in Dukes’ stages A to D, respectively. RNA was extracted and the concentrations of CEA mRNA copies and 18S rRNA in the extract determined by real-time qRT-PCR. Values are normalized by calculating the CEA mRNA copy/18S rRNA unit ratio. Each dot represents one lymph node.

Figure 12b Comparison between CEA mRNA expression levels in lymph nodes from the same patient. Selected data from Figure 12a. Results from patients from whom more than one lymph node was collected are shown. Individual lymph nodes are indicated by dots and lymph nodes from the same patient connected by vertical solid lines. Dukes’ stage of the tumor is indicated along the X-axis.
**Figure 12c** Selected data from Figure 12a. Each patient is represented by one lymph node with highest CEA mRNA level. (†) indicates patients who have died from cancer. (fat arrow) indicates patients with tumor recurrences. (broken arrow) indicates patients who received preoperative irradiation. Lymph nodes above the v-shaped line had tumor cells identified by routine hematoxylin/eosin staining. Horizontal bars indicate medians. Given P-values were obtained when comparing CEA mRNA levels in lymph nodes from non-cancer patients with the corresponding values of CRC patient groups with tumors in Dukes' stage A, B, C and D, respectively.

**Figure 12d** Comparison between CEA mRNA levels in the original tumor tissue (Tumor) and the lymph node with the highest CEA mRNA level (Lymph node) of 10 CRC patients with tumors in Dukes' stage B. The corresponding tumor tissue and lymph node values in each individual are connected by solid lines. CEA mRNA levels were determined as in (12a).
Comparison between CEA mRNA and CEA IHC.
A total of 96 lymph nodes from 42 CRC patients with tumours in Dukes’ stages A to D (n = 75) and 7 non-cancer patients (n = 21) were analysed both for CEA mRNA levels and presence of cells expressing the CEA protein by IHC and also by routine histopathologic examination. Nine lymph nodes had overt metastases. These were all positive in IHC (> 25 CEA+ cells/section) and had CEA mRNA levels above 5 copies/18S rRNA unit. Figure 13a. Sixty-six lymph nodes from CRC patients were judged as negative for tumour infiltration by routine histopathologic examination. Of these 23/66 showed positive staining with anti-CEA mAb in IHC. All had low frequency of positively stained cells (1 - 25 CEA+ cells/section; Figure 13a). CEA+ cells were only detected in one of the lymph nodes from controls. The positive node had one CEA+ cell (group 1 - 5 CEA+ cells in Figure 13a) and was from a patient with ulcerative colitis who had the highest CEA mRNA level in lymph nodes in the control group. Figure 12c. A large number of lymph nodes from CRC patients that were negative for infiltrating tumour cells both by routine histopathology and anti-CEA IHC had CEA mRNA levels above $10^2$ copies/18S rRNA unit. Figure 13a. All lymph nodes from non-cancer patients had CEA mRNA levels below this value. Using this as cut-off, all patients with tumours in Dukes’ stage C, more than half of the patients with tumours in Dukes’ stages B and D, and half of the patients with Dukes’ stage A tumours had at least one lymph node with elevated CEA mRNA level suggesting tumour cell dissemination. Table 4. No lymph node from non-cancer patients had > 5 CEA+ cells / section in IHC. Therefore > 5 CEA+ cells / section was used as the limit to judge a lymph node positive in IHC. The frequency of patients with lymph nodes positive in anti-CEA IHC was lower than that of patients with lymph nodes positive for CEA mRNA by qRT-PCR. Table 4. This difference was most pronounced in patients with tumours in Dukes’ stage B in which case only 20% had lymph nodes positive in anti-CEA IHC and while 65% had lymph nodes with increased CEA mRNA level (P=0.003).

Comparison between CEA mRNA and serum CEA
Preoperative serum samples were collected from 36 CRC patient and the serum concentration of CEA was determined by ELISA. No correlation was seen when the CEA concentration was compared with the CEA mRNA level in the lymph node with the highest CEA mRNA level in the individual patients. Figure 13b. Still patients with tumours in Dukes’ stages C (n = 7) and D (n = 7) had the highest CEA serum concentration (median 5 ng/ml in both patient groups compared to 1 ng/ml and 2 ng/ml in Dukes’ stages A and B respectively).

Using a cut-off point of > 2.5 ng/ml to one third of the patients with tumours in Dukes’ stage B were positive. Table 4. This was intermediate to the frequency of patients with Dukes’ B tumours that had elevated CEA mRNA levels in lymph nodes and those who had lymph nodes with CEA+ cells detected by IHC. Table 4. The two patients with Dukes’ B tumours who died from CRC both had high CEA mRNA levels in their lymph nodes. Figure 12c. Their CEA serum levels were also elevated although only marginally (4 and 6 ng/ml).
Table 4. Comparison between detection rate of CEA in patients with CRC by using different methods.

<table>
<thead>
<tr>
<th>Patient group</th>
<th>CEA mRNA in lymph nodes (&gt; 0.01 copies/18S rRNA U)</th>
<th>CEA+ cells in lymph nodes (&gt; 5 CEA+ cells/section)</th>
<th>CEA in serum (&gt; 2.5 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dukes’ A</td>
<td>3/6*</td>
<td>0/4</td>
<td>0/5</td>
</tr>
<tr>
<td>Dukes’ B</td>
<td>17/26</td>
<td>4/21</td>
<td>6/17</td>
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<td>Dukes’ C</td>
<td>10/10</td>
<td>7/10</td>
<td>5/7</td>
</tr>
<tr>
<td>Dukes’ D</td>
<td>6/9</td>
<td>5/7</td>
<td>4/7</td>
</tr>
</tbody>
</table>

* One to four regional lymph nodes were collected at the time of surgery and expression levels of CEA mRNA and 18S rRNA determined by real-time qRT-PCR. Patients with at least one lymph node with > 0.01 CEA mRNA copies/18S rRNA unit were regarded as positive.

° One to four regional lymph nodes were collected at the time of surgery and assayed for CEA expressing cells using CEA specific mAb in immunohistochemistry. One section/lymph node was inspected. Patients with at least one lymph node with > 5 CEA positive cells/section were regarded as positive.

# Peripheral blood was collected the morning before surgery and serum concentrations of CEA determined by ELISA. Patients with serum levels > 2.5 ng/ml were regarded as positive.

* Number of patients found positive by the indicated method/number of patients analyzed.

**Figure 13a** Comparison between CEA mRNA levels and frequency of cells expressing the CEA protein in lymph nodes. CEA mRNA levels were determined as described in Figure 12a. One section from the same lymph node was stained with the CEA specific mAb II-7 in IHC and the whole section inspected for positively stained cells. Positively stained sections were divided into three groups; sections with single or few scattered CEA+ cells (1 - 5), sections with one or several small aggregates of CEA+ cells (6 - 25) and sections with CEA+ cells in larger aggregates (> 25). Open circles indicate lymph nodes with tumor cells identified by routine hematoxylin/eosin staining and filled circles lymph nodes in which no tumor cells were detected by this method. The Figure includes results from analyses of both CRC and non-cancer patients.

**Figure 13b** Comparison between CEA mRNA levels in lymph nodes and concentration of CEA in preoperative serum of CRC patients with tumors in Dukes’ stage A to D. Serum was collected the same morning as the surgical procedure and CEA concentration was determined by ELISA. The CEA serum concentration is plotted against the value of the patient’s lymph node with the highest CEA mRNA level. CEA mRNA levels were determined as described in Figure 12a.
DISCUSSION

Paper I

We observed cytokeratin-positive cells in the lymph nodes among 47 of the 147 (32%) patients. This number is consistent with most studies using cytokeratin markers, even if figures ranging from 19% to 100% have been reported. We noticed no difference between patients with tumours in Dukes’ stage A or B (34% vs. 31%), but there was a significant difference between colon and rectal tumours (24% vs. 47%). In the survival analysis after 5 years and repeated after 10 years, we could not find any differences in the survival rate between those patients having and those lacking cytokeratin-positive cells in their resected lymph nodes. Thus, the findings of cytokeratin-positive cells did not imply upstaging of the tumours. The observation that both Dukes’ stage A and B tumours harbour a considerable number of cytokeratin-positive lymph nodes together with the significantly worse prognosis among patients in Dukes’ stage B contradicts the hypothesis that the presence of the cytokeratin-positive cells is indicative for metastatic disease. The same conclusion may be drawn because, although cytokeratin-positive cells were detected more often in rectal cancers than in colon cancers, there were no significant differences regarding either the number of recurrences or patient survival time between colon and rectal cancers.

It should also be remembered that 30-50% of patients with conventionally identified lymph node metastases (Dukes’ stage C) are cured by surgery alone removing the tumour together with the lymph nodes. The clinical significance of “micrometastasis” is still controversial. Nine studies using cytokeratin markers have been published after our study was completed. Four reported adverse outcome related to the findings of cytokeratin-positive cells, and five studies reported no difference in survival. Sasaki et al. and Yasuda et al. reported adverse outcome related to higher numbers and more distant location from the main tumour of cytokeratin positive lymph nodes, although all 19 studied patients had positive lymph nodes in the study of Sasaki and 76% of the patients in the study of Yasuda. Isaka et al. who studied 42 patients with rectal cancer in Dukes’ stage B, found no difference in survival after 5 years, but a significant difference at 10 years after operation. This was not the case in our study.

One single section from each lymph node was analysed similar to many studies. Analysis of an increased number of sections from lymph nodes, i.e. three sections, did not alter the prognostic significance as reported of Adell et al. In a study of Hitchcock el al. three levels in each lymph node separated by 20μm were studied. In the first section 64% of the lymph nodes containing cytokeratin-positive cells could be identified and another 36% in the second and/or third level. However, they did not study the impact on prognosis of the findings. Noura et al. present similar results; 23,5% of patients with cytokeratin-positive lymph nodes when studying one section increased to 45,9% of the patients when examining five sections. In spite of the increased sections included they could not find any prognostic value related to the presence of cytokeratin positive cells in the lymph nodes.

The nature of the cytokeratin-positive cells is obscure. The tumour cells observed may be dormant or without adequate requirements necessary for growth. The microscopic evaluation includes a cytologic interpretation to exclude false positive cells and cell debris. There have been speculations that the surgical manipulations could release cells to be transported to the lymph nodes. Tschmelitsch et al. studied 90 lymph nodes from 10 patients with non-malignant disease without any findings of cytokeratin-positive cells in the lymph nodes. We could verify this negative observation in 35 lymph nodes from 10
patients resected for benign colorectal disease.
We used the murine monoclonal anticytokeratin antibody CAM 5.2, which is one of the most widely used cytokeratin antibodies. This antibody, originally raised against the colon cancer cell line HT 29, recognises the cytoskeletal components cytokeratin 8 and 18. These cytokeratins have been described to be expressed in normal lymph node tissue and blood. Subsequently cytokeratin 20 has been proposed to be more specific for colon epithelial cells. The findings of negative control lymph nodes indicate that this may be a problem more in highly sensitive techniques like RT-PCR.

Paper II
We found, that the serum levels of free MMP-2, and total amounts of MMP-9, TIMP-1, and TIMP-2 were significantly higher in CRC patients than in controls, whereas the opposite observation was seen for the MMP-2/TIMP-2 complex. The background to these different relations of markers to controls is not obvious, but one possible explanation may be some defect in the complex formation in cancer patients, thus explaining the elevated MMP-2 level and decreased levels of MMP-2/TIMP-2 complex.

Our results are in line with the reports by Zucker et al.\textsuperscript{109} who reported elevated plasma levels of MMP-9 in CRC patients. Moreover, also in other malignancies elevated levels of MMPs have been reported. In gastric cancer Endo et al.\textsuperscript{170} observed elevated levels of both serum proMMP-2 and plasma proMMP-9, and Hayasaka et al.\textsuperscript{171} reported that MMP-9 plasma levels were significantly elevated in patients with hepatocellular carcinoma.

Also elevated levels of TIMPs have been reported in other studies. In a recent study Holten-Andersen et al.\textsuperscript{172} found high levels of total TIMP-1 in plasma from CRC patients including those with early-stage disease. They calculated a sensitivity of 63\% at 98\% specificity in colon cancer and the combination with CEA increased the sensitivity. Hence, they considered TIMP-1 as a marker for early detection of colon cancer patients. In a study on 90 lung cancer patients, Ylisirnio et al.\textsuperscript{173} using the same ELISA as we, reported elevated levels of serum TIMP-1 and lower levels of TIMP-2 and MMP-2/TIMP-2 complex than control subjects. High TIMP-1 levels or high MMP-9 correlated to poor prognosis and could serve as prognostic markers. Interesting to notice is that TIMP-1, apart from the strong inhibitory effect on various MMPs also has shown to be a growth-stimulating factor for normal and malignant cells.\textsuperscript{174}

Correlations to stage
We could not reveal any major value of the MMPs and TIMPs levels in identifying patients with metastatic disease, even if serum TIMP-2 was significantly higher in patients with than without distant metastases. This finding is opposite to the results in IHC studies of Ring et al.\textsuperscript{104}, showing that the TIMP-2 expression was higher in localized tumours than in tumours with regional or distant metastases. One possible explanation to this discrepancy may be that MMPs and TIMPs in localized tumours do not reach the blood pool to the same extent as do MMPs and TIMPs in metastases.

In the literature, we have found only three more studies describing a positive correlation between any of the analysed factors and the tumour stage. Yukawa et al.\textsuperscript{175} studied TIMP-1 in 54 patients with CRC for staging showing a significant association between plasma-levels and serosal invasion, liver metastases and Dukes’ stage C tumours. Their calculated cut-off value for plasma-TIMP-1 resulted in accuracy rate around 68\% in predicting these factors, which was better than for serum CEA and CA19-9. However, the range in sensitivity was 35-92\% and the specificity varied between 48-91\% dispite the use of two optimized cut-off levels for TIMP-1. Garbisa et al.\textsuperscript{176} found that serum
MMP-2 level was significantly higher in lung cancer patients with presence of distant metastases versus patients without tumour spread, and Jung et al.\textsuperscript{177} showed that patients with prostate cancer with metastases had significantly higher TIMP-1 plasma levels than patients without metastases.

The following studies have, however, reported pessimistic findings concerning the MMPs and TIMPs value for detection of metastatic disease. Zucker et al.\textsuperscript{109} showed an increase in MMP-9 plasma levels in colon cancer and breast cancer patients; Hayasaka et al.\textsuperscript{171} reported elevated plasma levels of MMP-9 in patients with hepatocellular carcinoma; Endo et al.\textsuperscript{170} observed increased levels of both serum proMMP-2 and plasma proMMP-9 in patients with gastric cancer, and Zucker et al.\textsuperscript{178} studied the MMP-2 plasma levels in patients with various types and stages of cancer – none of these studies could describe any significant differences between blood levels of analysed factors and tumour stage. Thus, we conclude that the value of reported MMPs and TIMPs analyses in blood samples for the discrimination between patients with or without distant metastases from various cancers must be considered as limited. Rather the elevations may reflect the cancer form and not tumour stage.

**Correlations to survival**
The survival analyses in all patients showed, that only elevated TIMP-2 correlated to poor survival time. If Q3 cut-off limit instead of the median cut-off limit was used in the survival analyses, none of the factors correlated to survival time. This demonstrates, that the selection of cut-off level is very important in the survival analyses. Far more interesting was the question whether MMPs and TIMPs blood levels could discriminate between patients in Dukes’ stages A-C that would die from their cancer and patients in Dukes’ stages A-C that were cured by surgery alone. However, the survival analyses in these potentially cured patients turned out to be of limited help in clinical decision making, because only elevated MMP-2, using the Q3 cut-off limit, correlated to poor survival time. Similar implications may, however, be made concerning the choice of cut-off level as were made above. Thus, in the present study on CRC no marker analysis could identify those patients that probably would have gained from receiving adjuvant chemotherapy or would have gained from intense surveillance.

**Clinical significance**
In our study, as well as in others, there were large overlapping ranges between serum levels of MMPs and TIMPs in patients and controls. When the reference limits were calculated, only 5-66% of the patients presented elevated levels of the analysed factors. These results considerably reduce the potential clinical use of blood analyses of MMPs and TIMPs. The observations that some of the control levels varied according to gender (MMP-9, TIMP-2), even if this was not revealed among the patients, certainly complicated the interpretation of the results. Furthermore, the findings that serum levels increased by age, as were shown for TIMP-1 and MMP-2/TIMP-2 complex in controls, contributed to the evaluation difficulties.

**Methodological aspects**
It has been discussed by others\textsuperscript{179} whether the levels of MMPs and TIMPs differs when analysed in serum or in plasma samples. It has been shown, that serum levels commonly are higher than plasma levels, and one given explanation to this is, that the factors are released during the clotting process. TIMP-1 has been shown to be produced and released from megakaryocytes and platelets.\textsuperscript{180} However, all controls and tumour samples used in the present study were serum samples, thereby eliminating discrepancies in the results between the two groups due to the processing.
We conclude, that the results presented in our study indicate, that the analysed MMPs and TIMPs do not contribute neither to tumour staging nor to prognosis in CRC. Thus, we cannot recommend that these serum analyses are to be included in the clinical routine.

**Paper III**

In the present study, including only patients with CRC Dukes’ stage C, we were able to show a possible importance of the immune system in the defence against malignancy. We quantified the number of two types of immune cells (CD8+ cytotoxic T-cells and CD68+ macrophages) in the cancer cell stroma of the regional lymph nodes in the surgical specimens. Expression of the R0 isoform of CD45 was used as a marker for activation. Being valid for all three stainings (CD8, CD45R0 and CD68), we observed significantly longer survival time for patients with high numbers of such positive cells in their regional lymph node metastases as compared to the remaining patients. These differences were still valid for CD8 and CD45R0 after exclusion of the patients that were given preoperative radiotherapy and/or postoperative chemotherapy. CD45R0 is expressed on activated and memory CD4+ and CD8+ T-cells. This means that the CD45R0+ and the CD8+ cells detected may be an overlapping population in certain patients, since this is the phenotype of effector cytotoxic T-cells. Survival analyses based on the combinations of two or all three CD stainings did not further increase the survival differences to any major extent. Our findings strongly support, that the numbers of immune cells in the regional lymph node metastases may serve as a potential adjunct when predicting the prognosis.

The prognostic implications of the presence of immune cells are in accordance with earlier studies on primary CRC by Jass et al.\textsuperscript{28} reporting a positive effect on survival time for patients with a pronounced lymphocytic infiltration at the tumour invasive margin. Moreover, in studies by Ropponen et al.\textsuperscript{86} and Naito et al.\textsuperscript{88} the numbers of tumour infiltrating lymphocytes (TILs) were shown to be an independent prognostic factor of survival for patients in all stages of CRC. However, there is no other study to be found reporting on immune cells in regional lymph node metastases according to prognosis.

There are as yet no methods available for selection of the 50-70% of the CRC patients operated on for a Dukes’ stage C tumour, who later on will die from tumour relapse. This implies, that many patients will unnecessarily be treated with chemotherapy, a therapy causing side-effects, and demanding repetitive administrations during at least half a year to considerable discomfort and large expences. Thus, we consider our observations to be of interest not only for prognosis, but we also believe that further clinical trials with adjuvant immunostimulatory therapy alone in combination with traditional chemotherapy (5-Fluorouracil + Leucovorin) will be justified.

**Paper IV**

We demonstrated that CEA mRNA, with its highly restricted expression pattern limited to epithelial cells predominantly of the large bowel and CRC while not being expressed in immune cells\textsuperscript{40} is a useful marker for disseminated tumour cells. The identification of CEA expressing cells in lymph nodes, e.g. outside epithelial compartments, was not complicated by the expression of highly homologous CEA gene family members present in immune cells. Four CEA-related macromolecules, i.e. BGP, NCA, CGM1 and CGM6, are expressed at high levels in neutrophil granulocytes. BGP is also expressed in activated lymphocytes\textsuperscript{181-183} likely to be numerous in lymph nodes. The mRNA 3’-untranslated region (3’-UTR) has the least homology between the different CEA gene family members.\textsuperscript{181} In this region we were able to find sequences in different exons of
the CEA gene (CEACAM5) that allowed selection of one combination of primers and probe that was suitable for real-time qRT-PCR and at the same time was highly selective for CEA mRNA.

There are presently two detection methods for real-time qRT-PCR available. One utilizes a fluorocrome labeled internal probe that hybridizes to a sequence in the amplicon and gives a signal upon degradation by the polymerase. The other method of detection relies on fluorescent dyes like Syber Green I, which selectively bind to dsDNA. The first method ascertains that only correct PCR amplification is monitored, while it is not possible to discriminate between correct and illegitimate PCR products by the second method. The present assay utilizes the first method, which most likely contributed to the high specificity of the assay, allowing detection of CEA mRNA in the presence of excess of potentially cross-reactive mRNA species from immune cells. The assay is also quite sensitive and gives reliable signals down to 20 CEA mRNA copies, which corresponds to approximately 2 LS174T colon cancer cells. The sensitivity of the assay is influenced by the proportion of the mRNA species of interest in the RNA sample. In order to mimic the conditions with only a few tumour cells present in a lymph node we therefore diluted the RNA copy standard in buffer containing excess of RNA extracted from PBMC. Furthermore, we found that utilizing the 3'-primer for specific reverse transcription, so called TaqManEZ technology, increased the chances of getting a specific signal from CEA mRNA when present in low proportion more than 10-fold compared to preparation of cDNA using random hexamers followed by specific PCR amplification. This was true even if the highly efficient AmpliTaqGOLD DNA polymerase was utilized in the PCR (data not shown).

PBMC samples from healthy controls and lymph nodes from patients with benign disease had less than 10^(-2) CEA mRNA copies/18S rRNA, which corresponds to ≈ 1 tumour cell per 10^5 immune cells as judged from experiments with LS174T cells admixed to PBMC. However, CEA mRNA was detected in two thirds (23/34) of the control lymph nodes and only in one third (4/13) of the PBMC samples. The average CEA mRNA level was slightly higher in lymph nodes of control patients (median 8 x 10^(-5) copies/18S rRNA unit) compared to PBMC (median < 10^(-5) copies/18S rRNA unit). This might reflect a minor release of epithelial cells to the lymphatics also in benign conditions, particularly in ulcerative colitis, where changes in the colonic epithelium is a prominent feature. In previous studies, different groups have come to different conclusions for the utility of CEA mRNA as a marker for disseminated tumour cells using qualitative RT-PCR for detection. CEA mRNA was demonstrated in healthy controls in some studies but not in others. The discrepancy may be explained by differences in both sensitivity and specificity of the assays. Using qRT-PCR it is possible to set a cut-off point for significant CEA mRNA expression. Therefore, occasional low CEA mRNA expression in controls is not an obstacle in estimating tumour cell dissemination. Regardless of the tumour stage, the majority of patients with CRC had at least one lymph node with higher CEA mRNA level than controls. The CEA levels in the group of patients with Dukes' stage B tumours did not show a Gaussian distribution, suggesting heterogeneity in lymph node involvement. Thus, patients with tumours in Dukes' stages A and B showing the highest levels of CEA mRNA may constitute the patients at risk of tumour recurrences. Indeed two of the patients with high CEA mRNA level and Dukes' B tumours have died from cancer. The clinical cut-off level, however, remains to be determined in prospective studies with longer follow-up.

Occasionally there were significant differences in CEA mRNA levels between
lymph nodes from the same resected specimen. Thus, variation in CEA mRNA levels could partly reflect problems in identifying the primary draining lymph nodes where metastatic cells are most likely to be found. The evolving technique using the sentinel node concept can be utilized in identification of the adequate lymph nodes thereby reducing the total work-load. Even though the risk of false negative sentinel nodes, so called skip lymph nodes is a major concern, the method is likely to improve the sampling procedure. Certainly, the method needs to be evaluated in larger prospective trials.

Lymph nodes positive in routine hematoxylin/eosin staining had CEA mRNA levels corresponding to more than 1 tumour cell per 100 immune cells as estimated from admixing LS174T cells in PBMC. CEA mRNA levels in the original tumour tissue varied 25-fold and cells of the well-differentiated colon carcinoma cell line LS174T had approximately 20-fold higher expression level of CEA mRNA compared with cells of the less differentiated HT29 line (data not shown). Thus, it is likely that the lower values represent lymph nodes in which more than every tenth cell is a tumour cell albeit these have the lower expression level of CEA mRNA. Differences in CEA mRNA expression level most likely reflects differences in differentiation stage of the tumour since CEA mRNA expression increases with differentiation in normal colonic epithelial cells. The observed high CEA mRNA levels in lymph nodes with overt metastases are expected and do not imply changes in therapeutic strategies.

Various techniques have been applied for detection of tumour cells in regional lymph nodes. These include fat-clearing techniques for retrieval of higher numbers of lymph nodes combined with classical histologic examination and serial sectioning of lymph nodes in histopathology. These two methods have not proven ideal in detection of tumour dissemination. IHC using tumour marker specific mAb and determination of mRNA for selected tumour marker proteins by qualitative RT-PCR have also been used for detection of disseminating tumour cells. One study analyzed lymph nodes from 7 CRC patients and showed that quantitative RT-PCR based on selective binding of fluorescence dye to dsDNA was more sensitive than qualitative RT-PCR for detection of CEA mRNA. Anti-CEA and anti-cytokeratin (8/18, 19, or 20) staining has been used for detection of tumour cells in regional lymph nodes of CRC patients, and the impact of disseminated tumour cells on prognosis has been evaluated. The results are conflicting. The method has also been questioned due to limited reproducibility even when the sensitivity was increased by analysis of multiple sections. In this study, we confirmed the relatively low sensitivity of anti-CEA IHC, and moreover, we showed a great overlap in CEA mRNA levels between lymph nodes with up to 25 CEA positive cells / section and nodes with no detectable CEA positive cells. Furthermore, some lymph nodes in which CEA positive cells were detected had low CEA mRNA levels and one even had CEA mRNA levels below the detection limit. These observations suggest, that cells positive in IHC might be dead or apoptotic cells that are no longer producing the protein but still express it. That protein expression can continue after mRNA expression has ceased was shown for BGP in normal colonic epithelial cells. It has been demonstrated that macrophages located in the proximity of the colonic epithelium can have phagosomes containing debris of epithelial cells staining positive for epithelial antigens. Moreover, studies in rat have shown that dendritic cells migrate between the intestinal lamina propria and the regional lymph nodes thereby transporting components from apoptotic normal epithelial and tumour cells to the T cell area of the nodes. These findings establish the possibility, that CEA positive cells in lymph nodes may even be macrophages.
containing CEA taken up by phagocytosis. Thus, a cell positively stained for the CEA protein is not necessarily an indication of a viable tumour cell that may proliferate and disseminate, whereas the presence of CEA mRNA most likely is. Taken together, these results argue against using occasional CEA positive cells by IHC as a selection criterion for adjuvant therapy. The fact that several lymph nodes with relatively high CEA mRNA levels were negative for CEA in IHC most probably reflects reduced sampling error as the probability of finding a tumour cells in a tissue section, which constitute far less than 1% of the volume of the lymph node, is low compared to the probability of detecting CEA mRNA in RNA extracted from half the volume of a lymph node.

The CEA mRNA levels in lymph nodes also seem superior to the concentration of the CEA protein in serum as a marker for disseminated tumour cells. The two patients with tumours in Dukes’ stage B who died from their cancer had elevated serum CEA concentration in agreement with the suggestion of elevated serum CEA as a prognostic parameter in CRC. However, their serum levels of CEA was close to the cut-off value for positivity while their CEA mRNA levels in lymph nodes were among the highest in the Dukes’ stage B tumours, suggesting that the CEA mRNA level might be of better prognostic value than the CEA serum concentration. This is in accordance with the study by Guadagni et al., who reported that CEA mRNA may prove better than CEA protein as a prognostic indicator in blood.

The clinical significance of ectopic finding of CEA mRNA in lymph nodes and, hence, presumed disseminated malignant cells remains to be proven. Liefers et al. analyzed lymph nodes from 26 stage II CRC patients for presence of CEA mRNA using qualitative RT-PCR. The cancer-specific survival rate was 91% in node negative patients compared to 50% for patients positive for CEA mRNA in their lymph nodes. Rosenberg et al. reported a tendency towards shorter disease free survival for CRC patients with CEA mRNA positive lymph nodes, but their findings were not statistically significant. Godfrey et al. have shown a prognostic value of CEA mRNA positivity in lymph nodes in a study of node-negative esophageal cancer, using quantitative RT-PCR. Nine of eleven patients with lymph nodes positive for CEA mRNA had recurrences compared to one of nineteen of patients with negative lymph nodes.

We have been able to demonstrate, that CEA mRNA as detected by the qRT-PCR technique, is indicated as being superior to other conventional methods used for tumour staging in CRC. If the observations can be proven in extended studied on clinical materials with long follow-up, this method is of utmost interest when selecting patients to adjuvant therapy.
CONCLUSIONS

Cytokeratin 8/18 positive cells in regional lymph nodes in the resected specimens are frequent findings in Dukes’ stage A and B CRC, but in our study of no prognostic value.

Preoperative serum-levels of free MMP-2, MMP-2/TIMP-2 complex and total amounts of MMP-9, TIMP-1 and TIMP-2 are of limited value for tumour staging and prognosis in CRC.

High numbers of CD8+ , CD45R0+ and CD68+ immune cells in regional lymph node metastases may serve as a positive prognostic factor in Dukes’ stage C CRC.

Quantitative RT-PCR detecting CEA mRNA in regional lymph nodes is a highly sensitive and specific diagnostic tool in detecting tumour cells. The clinical impact remains to be evaluated in further studies.

In summary, our studies aimed to identify methods for improved tumour staging in CRC indicate, that real-time quantitative RT-PCR for CEA seems to be a more sensitive method than immunohistologic detection of CEA-positive cells for early detection of disseminated disease in the regional lymph nodes.
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