Experimental and population-based studies on colorectal cancer

Thymidine phosphorylase as a potential biomarker

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To Kristoffer and Greger

Two truths approach each other. One comes from within,
one comes from without—and where they meet you have the chance
to catch a look at yourself.

— ***Tomas Tranströmer***

ABSTRACT

Experimental and population-based studies on colorectal cancer – Thymidine phosphorylase as a potential biomarker

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Background: Colorectal cancer (CRC) is one of the most common malignancies, and the only reliable treatment option for cure is surgery.

Method: Quantitative real-time polymerase chain reaction was used to analyze the gene expression of the enzyme thymidine phosphorylase (TP), which was related to prognostic factors (paper I, n=254), evaluated as a predictive factor (paper III, n=125), and assessed by change of treatment (paper II, n=28). Data from the Swedish Colorectal Cancer Registry were retrieved and analyzed in order to assess adherence to present clinical guidelines (n=34,000).

Results: In stage III CRC, TP analyzed in tumor tissue correlated with lymph node staging, with higher expression levels relating to a greater number of positive nodes and a worse N-stage. Higher TP expression was also associated with a worse histological tumor grade. Rectal cancer exhibited significantly higher TP expression in mucosa and tumor tissue compared to colon cancer. There was a significant increase of TP gene expression when comparing rectal cancer biopsies before and after radiotherapy. In addition, a decrease in TP levels was noted after chemotherapy. In patients with metastatic CRC, time to progression was significantly longer in patients with high TP expression, but there was no correlation to tumor response rate or palliative survival.

The factors associated with adherence to guideline treatment in colon cancer stage III patients were lower age, less comorbidity, worse N-stage, and presence of a multidisciplinary team conference. One-third of the patients started their adjuvant chemotherapy more than eight weeks after surgery.

Conclusion: TP may be useful in prognostic and predictive situations. TP is affected by radiotherapy, which might be used in clinical settings. However, there are conflicting results, and TP and the methods of analyzing TP need to be evaluated further in larger studies. The adherence to guideline treatment in colon cancer stage III is acceptable in younger and healthier patients. In addition, there is scope for shortening the waiting time until the start of chemotherapy.

Keywords: colorectal neoplasms, thymidine phosphorylase, chemotherapy, adjuvant, biological markers, prognosis, practice guidelines, radiotherapy

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SAMMANFATTNING PÅ SVENSKA

Tjock- och ändtarmscancer är en av våra vanligaste cancersjukdomar som drabbar nästan 6000 personer om året i Sverige. Enda säkert botande behandling är kirurgi, medan cytostatika och strålbehandling kan komplettera kirurgin som tilläggsbehandling för att förbättra chanserna till bot. Cytostatika ges för att minska risken för att patienterna utvecklar dottertumörer. Då biverkningarna av cytostatika kan vara svåra är det viktigt att identifiera patienter med störst risk för återfall. Idag erhåller framförallt patienter där man finner tumörceller i lymfkörtlarna tilläggsbehandling med cytostatika. I *delarbete IV* utvärderas hur vi i Sverige följer de riktlinjer som idag finns för cytostatikabehandling vid tjock- och ändtarmscancer. Konklusionen är att riktlinjerna följs bra för yngre och friskare patienter, men att grupper finns där följsamheten är sämre. Man kan t.ex. se att patienter som har diskuterats på en multidisciplinär konferens oftare blir rekommenderade cytostatikabehandling. För att få bästa möjliga effekt av given behandling bör man också kunna förkorta tiden från kirurgi till start av cytostatikabehandling.

I *delarbete I–III* analyseras genuttrycket av enzymet tymidinfosforylas (TP) i vävnad från patienter med tjock- eller ändtarmscancer. TP har flera tumörfrämjande egenskaper, men är också är involverat i aktiveringen av cytostatika. I *delarbete I* korreleras TP till kända faktorer relaterade till en ökad risk för återfall i sjukdomen. Högt TP var förenat med växt i fler lymfkörtlar och mer omogna tumörceller. Fynden kan vara av kliniskt värde då man skulle kunna analysera TP i ett tumörprov taget före operation för att identifiera högriskpatienter som skulle kunna ha nytta av tilläggsbehandling före kirurgi. I *delarbete II* påvisas att genuttrycket av TP ökar efter given strålbehandling. Fyndet är av värde vid tolkningen av TP, men skulle också kunna vara kliniskt intressant då TP aktiverar cytostatika som används vid tjock- och ändtarmscancer. I *delarbete III* utvärderas sambandet mellan TP och hur väl en tumör svarar på given behandling. Högt TP var förenat med längre tid till att sjukdomen framskrider från det att cytostatikabehandlingen påbörjades. Det fanns dock inget samband med om dottertumörerna röntgenmässigt svarade på behandlingen eller hur lång tid det var från start av behandling till död.

Med ökad kunskap om prognostiska faktorer och faktorer som förutspår hur en tumör kommer svara på behandling finns en bättre möjlighet att kunna individualisera och optimera den enskilda patientens behandling. TP kan vara av intresse, men resultaten i denna avhandling behöver bekräftas i större studier, då motstridiga resultat finns.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Lindskog EB**, Wettergren Y, Odin E, Gustavsson B, and Derwinger K
Thymidine phosphorylase gene expression in stage III colorectal cancer.
Clin Med Insights Oncol. 2012; 6: 347-353.

II. Derwinger K, **Bexe Lindskog E**, Palmqvist E, and Wettergren Y.
Changes in thymidine phosphorylase gene expression related to treatment of rectal cancer.
Anticancer Res. 2013; 33(6): 2447-51.

III. **Bexe Lindskog E**, Derwinger K, Gustavsson B, Falk P, and Wettergren Y
Thymidine phosphorylase expression is associated with time to progression in patients with metastatic colorectal cancer.
BMC Clin Pathol. 2014; 14: 25.

IV. **Bexe Lindskog E**, Ásta Gunnarsdóttir K, Derwinger K, Wettergren Y, Glimelius B, and Kodeda K.
A population-based cohort study on adherence to practice guidelines for adjuvant chemotherapy in colorectal cancer
Submitted for publication

ABBREVIATIONS

cDNA	Complementary DNA
CRC	Colorectal cancer
CT	Computed tomography
2-DDR-1P	2-deoxy-D-ribose-1-phosphate
DFS	Disease-free survival
DNA	Deoxyribonucleic acid
ECOG	Eastern cooperative oncology group
ELISA	Enzyme-linked immunosorbent assay
EORTC	The European Organisation for Research and Treatment of Cancer
FLV	5-fluorouracil and leucovorin
5-FU	5-fluorouracil
Gy	Gray, the absorption of one joule of energy by one kilogram of matter
IHC	Immunohistochemistry
LNR	Lymph node ratio
MDT	Multidisciplinary team
MNGIE	Mitochondrial neurogastrointestinal encephalopathy
MRI	Magnetic resonance imaging
mTP	Mucosa thymidine phosphorylase
PD-ECGF	Platelet-derived endothelial growth factor
sTP	Serum thymidine phosphorylase
SCRCR	The Swedish Colorectal Cancer Registry
TNM	Tumor, node, metastasis
TP	Thymidine phosphorylase
tTP	Tumor thymidine phosphorylase
TYMP	Thymidine phosphorylase
SCRCR	The Swedish Colorectal Cancer Registry
VEGF	Vascular endothelial growth factor

TABLE OF CONTENTS

1 INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies in Europe and one of the leading causes of cancer deaths worldwide.[1,2] In Sweden, about 6,000 people a year are diagnosed with CRC. Colon cancer is more common than rectal and accounts for two-thirds of CRC cases. Surgery offers the most reliable chance for cure. Modern radiotherapy and chemotherapy in addition to surgery improve survival. Even so, CRC is associated with a significant risk of premature death. To improve the outcome of patients with CRC, the hunt for better treatments goes on. Prognostic factors for identifying high-risk patients are important, and equally important are predictive factors that can predetermine the effects of specific treatments on individual patients.

There are no established prognostic or predictive biomarkers for CRC used in the everyday clinical setting. We currently rely on a prognosis based on the anatomically based TNM classification and a histopathological examination, together with patient-related factors such as performance status and circumstances related to surgery. Some patients receive chemotherapy treatment when they do not need it, and other patients receive treatments that have no effect on their particular tumors; meanwhile, they are exposed to the risk of serious side effects. The need to find prognostic as well as predictive markers is huge, and as treatment options become more and more complex, their identification will become even more necessary.

The background of this thesis was a previous investigation of 18 chemotherapy-related genes in which the gene expression of thymidine phosphorylase (TP) showed both a prognostic and a predictive value in patients with advanced CRC.[3] This thesis further evaluates TP as a potential biomarker in the scenario of CRC (papers I–III).

In terms of prognosis and prediction of appropriate chemotherapies, the question is, “How do we use the knowledge that we have today?” That concept is the background for the fourth manuscript.

1.1 The patient

CRC is more common with advanced age, and there are no large differences in incidence between genders. There are often no symptoms of early CRC,

and they then become diffuse. As a tumor evolves, one might notice a change in bowel habits. Diarrhea and constipation are possible symptoms. Anemia, unexplained weight loss, persistent abdominal discomfort, and accompanied fatigue are other symptoms that might bring a patient to the doctor's office. However, there are symptoms that result in a more acute onset; for instance, perforations result in acute abdominal pain, and complete bowel obstruction may cause interval pain, a large, bloated belly, and occasional vomiting. Significant bleeding from the tumor is another symptom that might bring the patient to the emergency department.

1.2 Diagnosis

When CRC is suspected, the workup starts with a patient history, a physical examination, and a digital rectal examination. Then, a fecal occult blood test is conducted to detect bleeding that is not visible to the eye. The rectum is easily examined further by rectoscopy; this exam is obligatory in order to determine the level and height of a rectal tumor. The rest of the bowel is examined by colonoscopy or computed tomographic colonography. Prior to surgery, additional examinations with computed tomography (CT) of the thorax and abdomen are included to exclude metastasis. Refined diagnostic tests of the liver may be necessary, for which contrast-enhanced magnetic resonance imaging (MRI) or ultrasound are options. The preoperative workup for rectal cancer also includes an MRI of the pelvic region, and sometimes a transrectal ultrasound, to determine the preoperative stage. At the preoperative multidisciplinary (MDT) conference, it is decided whether the patient is offered preoperative radiotherapy and chemotherapy. The decision is based on the preoperative tumor stage and whether the tumor is deemed resectable. Selected cases may also be candidates for a positron emission tomography scan.

1.3 Prognosis

Prognostic factors refer to the probability of future events in patients who currently have a disease; the information is usually applicable at the group or population level. The presence or absence of such prognostic factors or markers can be helpful in the selection of patients for treatment; however, prognostic factors do not predict responses to treatment.

The TNM classification, which is the cornerstone for making a prognosis, is currently the standard worldwide.[4] The anatomically based TNM classification uses the local, regional, and distant extents of the cancer to describe the disease.

1.3.1 Staging

The T-stage represents the local tumor stage and the extent of the tumor's invasion through the bowel wall.

TX: Primary tumor cannot be assessed.

T1: The cancer has grown into the submucosa.

T2: The cancer has grown through the submucosa and into the muscularis propria.

T3: The cancer has grown through the muscularis propria and into the subserosa, but not to any neighboring organs or tissues.

T4a: The cancer has grown through the subserosa.

T4b: The cancer has grown through the subserosa and is attached to or invades nearby tissues or organs.

The N-stage represents lymph node involvement.

NX: Regional lymph nodes cannot be assessed because of incomplete information.

N0: No cancer exists in regional lymph nodes.

N1a: Cancer cells are in one regional lymph node.

N1b: Cancer cells are in two to three regional lymph nodes.

N1c: Small deposits of cancer cells are found in the region, but not in the lymph nodes themselves.

N2a: Cancer cells are in four to six regional lymph nodes.

N2b: Cancer cells are in seven or more regional lymph nodes.

The M-stage represents distant metastasis.

M0: No distant metastasis exists.

M1: Distant metastasis exists.

M1a: Metastasis is confined to one organ.

M1b: Metastasis is in more than one organ or in the peritoneum.

Once a patient's T, N, and M classification has been determined, usually after X-ray, surgery, and completed pathology, the information is unified in a stage grouping process. This combined risk assessment based on the TNM classification separates patients into stages I, II, III, and IV (see Table 1).[4] Treatment and follow-up guidelines are often based on the overall stage group, illustrated in Figure 1 and specified in Table 1. The N-stage, which also reflects the number of positive nodes, is the main factor that determines whether the guidelines recommend adjuvant chemotherapy in CRC cases.[5]

Table 1. Stage by TNM 7TH edition

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

From UICC TNM 7th edition, 2009. Abbreviations: T=tumor; N= node, M= metastasis.

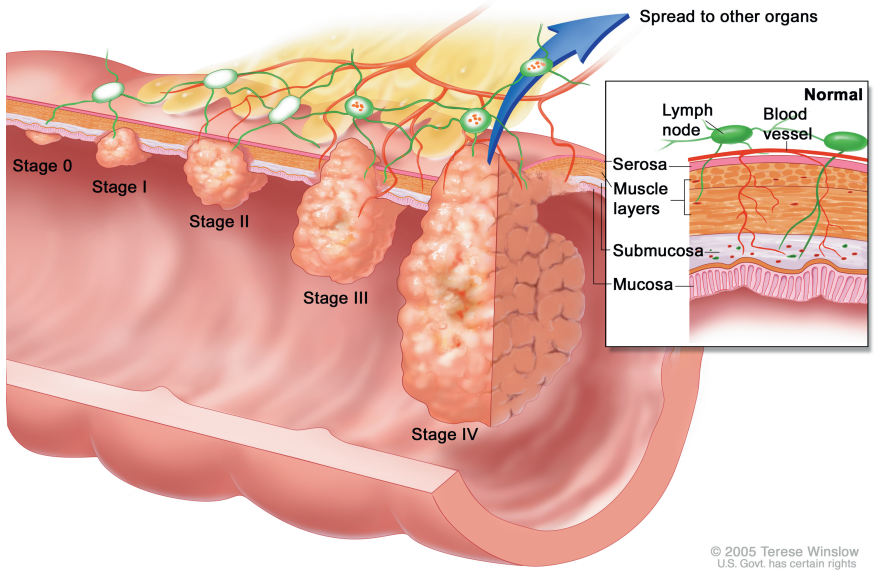

Figure 1. *Illustration of stages and tumor sizes in colorectal cancer. Reprinted by permission from illustration. For the National Cancer Institute © 2005 Terese Winslow; the U.S. Govt. has certain rights.*

1.3.2 Histopathological grading

Tumor differentiation is a stage-independent prognostic factor.[6,7] The differentiation grade is a description of how closely the cancer resembles normal colorectal tissue when looked at under a microscope. The grading system uses G1 (well differentiated), G2 (moderately differentiated), G3 (poorly differentiated), and G4 (undifferentiated); however, today they are often dichotomized to low grade (G1–G2) and high grade (G3–G4).

High-risk stage II

In stage II, there are patients with a good prognosis but also patients with a worse prognosis, who might benefit from adjuvant chemotherapy. To help define these patients, additional factors have been added. When one or several of these factors are present, stage II is defined as high risk. Such high-risk patients include those who have been operated on for emergency intestinal occlusion or perforation.[8,9] An inadequate examination of the lymph nodes in the specimen, a T4 tumor or poorly differentiated tumor, and

vascular or perineural invasions are other high-risk criteria.[5] However, with improved surgical techniques and better pathological examinations of surgical specimens, stage migration is a factor that is likely to lead to a better prognosis in stages II and III. Thus, a reevaluation of some of the high-risk criteria in stage II is probably necessary.

1.4 Treatment

1.4.1 Surgery

The main aim of surgery is cure with radical removal of the cancer. Occasionally, small tumors with no visible engagement of any lymph nodes are suited for local excision. Then, however, information about lymph node engagement is lost. Thus, the tumor may be more advanced than suspected, requiring additional surgery. Depending on the location of the colon cancer, different surgical techniques are performed. However, the aim is the same: radical resection of the tumor with good resection margins on both sides of the tumor and a high enough ligature of the vessel, which most often means one vascular arcade away from the tumor.

Surgery for rectal cancer is more complex because of the natural limitations of the pelvis and the proximity to sensitive structures, such as nerve bundles. Surgeons must consider not only the bowel margin at each end but also the circumferential margin. Radical surgery is the primary priority, but efforts are made to try to retain good quality of life and to preserve sensory and executive functions if possible.

1.4.2 Preoperative treatment

Patients with colon cancer typically go straight to surgery without any given preoperative treatment. Currently, some researchers are suggesting that a better approach might be to provide adequate systemic treatment prior to surgery.[10-12] The reasoning behind this concept is that patients are in a better physical status, which might allow them to tolerate the treatment better than after surgery, when the inflammatory cascade is initiated. Early treatment might also increase the chances of wiping out cancer cells outside of the primary tumor, such as micrometastases in the lymph nodes.

In treating rectal cancer, there is a routine with radiotherapy, depending on the preoperative stage of the tumor. Patients having early cancer with a clear margin goes straight to surgery, while those with locally advanced rectal cancers receive locally applied short-course radiotherapy with 5 Gray (Gy) for five consecutive days (5x5 Gy). Tumors assessed with a risk of surgery not becoming radical — i.e., many T4 tumors, node positive, and primarily inoperable rectal cancer — may receive long-course radiotherapy combined with chemotherapy.

1.4.3 Chemotherapy

Adjuvant chemotherapy

Surgery is the main curative treatment for CRC. With the support of additional chemotherapy, the chances of survival increase. The goal of adjuvant chemotherapy is to eradicate micrometastases that may have been left after radical resection of the tumor. The relative risk of relapse after radical resection of a primary CRC varies, depending on stage and presence of other clinical and pathological risk characteristics. The risk reduction offered by chemotherapy on a group level is highest in tumors with a worse primary prognosis. For example, the absolute risk reduction of death with 5-FU as single agent is 3–5% in stage II, but in stage III, a combination of 5-FU + oxaliplatin renders a risk reduction of 15–20%.[13] During the past few years, there has been a stage migration due to better pathological classification, which means that patients that used to be classified as stage II might now be classified as having stage III disease, resulting in a better prognosis in both groups, but not in a better prognosis overall (the "Will Rogers phenomenon").

Swedish guidelines recommend FLV (5-FU and leucovorin) or capecitabine as a single agent or in combination with oxaliplatin for stage III colon cancer; patients with high-risk stage II colon cancer may receive the same treatment as those with stage III.[14]

Chemotherapy is not recommended for rectal cancer in current Swedish guidelines. Nevertheless, although the scientific evidence is lower, European and US guidelines do recommend chemotherapy for rectal cancer.[13,15,16] At the 2013 European Registration of Cancer Care consensus conference, minimal or no consensus was reached regarding adjuvant chemotherapy for rectal cancer.[17] The Swedish guidelines are currently under revision and will

likely result in recommendations more similar to those for colon cancer in patients who have not received preoperative chemoradiation.

Palliative chemotherapy

Depending on the aim of the treatment, there are different approaches available for chemotherapy in metastatic disease. Treatments vary depending on the localization of the metastasis, whether there is a single or multiple metastases affecting one organ or several organs, and if there is a possibility of a secondary curative resection. Patient-related factors include biological age, performance status, comorbidities, and patient preferences. First-line chemotherapy treatment may be given with a curative intent before a clearly radical resection is planned or in cases where the metastases might become resectable after tumor shrinking. In the latter case, maximum tumor shrinkage is of importance, and a three- or four-drug combination may be applicable.[13] This highly intensive approach may also be suitable when there is a need for rapid tumor shrinkage to control symptoms or to achieve tumor control in a rapidly deteriorating disease with a high tumor burden. For patients with a low tumor burden, no symptoms, or high comorbidity, a more palliative approach with a low-toxic single- or two-drug regime is the first choice.[13] However, asymptomatic patients with low comorbidity and an unresectable disease might benefit from a more intensive first-line treatment.[18]

Available chemotherapy drugs for metastatic CRC are the fluoropyrimidines (5-FU, capecitabine), oxaliplatin, and irinotecan.[13,14,18] Additional treatment options are the vascular endothelial growth factor (VEGF) antibody bevacizumab and, for KRAS wild-type tumors, the epidermal growth factor receptor antibodies cetuximab and panitumumab.[13,14,18]

1.4.4 Radiotherapy

MRI results and other clinical information are used to classify rectal cancer preoperatively as staged early (good), intermediate (bad), or locally advanced (ugly) at MDT conferences. Early rectal cancers are those in which no preoperative radiotherapy is needed for a radical resection of the tumor. Tumors with a higher risk of local recurrence are deemed as intermediate, and radiotherapy with 5x5 Gy the week before surgery is recommended, although there is a risk of side effects. Locally advanced tumors include those that are primarily unresectable and those in which the risk of local recurrence is too high for primary surgery. Then, long-course radiotherapy as a single treatment or, most often, in combination with chemotherapy, is applicable.

Radiotherapy aims for a tumoricidal effect in the infiltrative zone close to the tumor and a tumor shrinking effect.

1.5 Thymidine phosphorylase

The correct nomenclature for thymidine phosphorylase is TYMP, but for simplicity,the more commonly used abbreviation, TP, will be used throughout the text. TP is an enzyme (E.C. 2.4.2.4) first discovered in 1954 for its involvement in the pyrimidine salvage pathway (Figures 2 and 3).[19] In 1987, the protein known as platelet-derived endothelial growth factor (PD-ECGF) was discovered; this protein is involved in and thought to stimulate endothelial cell growth. PD-ECGF was later shown to be identical to TP. TP is mainly an intracellular protein present in both the nucleus and the cytoplasm; however, TP's metabolite, 2-deoxy-D-ribose-1-phospate (2-DDR-1P), is secreted into the extracellular space.[20]

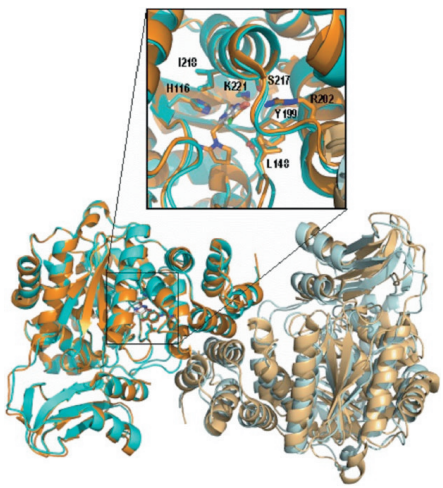

Figure 2. *Thymidine phosphorylase is an enzyme with a dimeric structure; active sites are boxed. Reprinted by permission from John Wiley and Sons.*

The gene encoding the TP protein is located on chromosome 22q13. TP mutations resulting in a loss of function have been identified as the cause of mitochondrial neurogastrointestinal encephalopathy (MNGIE). A reduction of TP activity results in an increase of thymidine and 2´-deoxyuridine levels, which can cause an imbalance of the mitochondrial nucleoside and nucleotide pools. Symptoms associated with MNGIE are weight loss, progressive gastrointestinal dysmotility, and motor and sensory neuropathy. TP is present in several normal tissues and cells; for example, TP levels are high in

platelets, macrophages, stromal cells, some epithelial cells, the endometrium, the bladder, and the lungs.

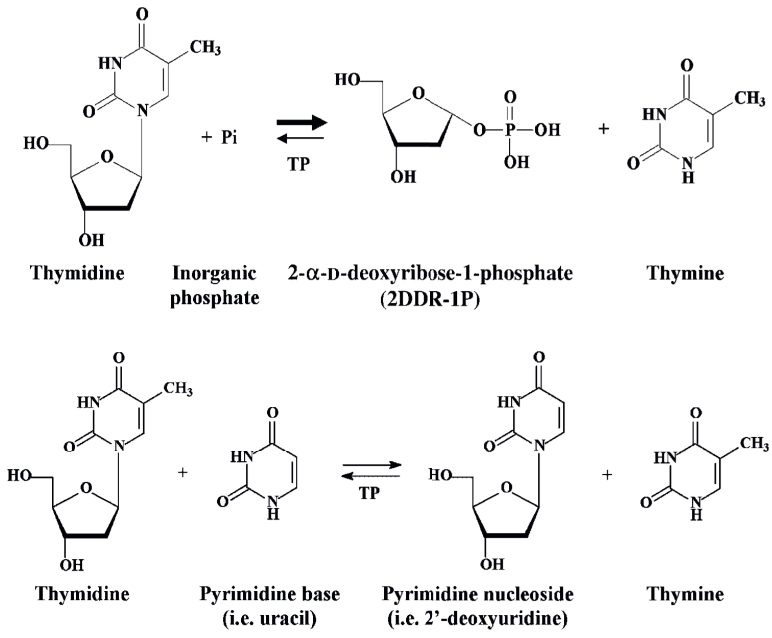

Figure 3. *Thymidine phosphorylase (TP) catalyzes the reversible conversion of thymidine to thymine and deoxyribose-monophosphate in the cell. TP also has the ability to transfer deoxyribosyl from one base to another. Reprinted by permission from John Wiley and Sons.*

It has been shown that TP levels are increased in several solid tumors, such as breast, bladder, gastric, colorectal, lung, esophageal, and thyroid tumors, compared with normal tissue.[21,22] In colorectal carcinomas, TP is expressed in epithelial cells, but also in stromal cells such as macrophages, endothelial cells, fibroblasts, and lymphocytes.[19,23-27] The expression is especially high in the tumor-infiltrating macrophages.[27]. In tumors there are macrophages that do not exert the same tumoricidal and bactericidal activity as classical macrophages do. Instead, tumor associated macrophages (TAMs) secrete several growth factors and anti-inflammatory cytokines, thus exhibiting tumor-promoting properties.[28,29]

A few studies have reported elevated TP levels in the plasma of cancer patients.[30,31] In addition to the possible prognostic value of TP levels in serum or plasma, a relationship between TP levels and response to chemotherapy in

esophageal and uterine cervical cancer has been discovered.[31,32] In a CRC study that measured TP levels in perioperative venous blood drainage from the tumor, it was found that high TP levels were correlated with a worse prognosis, in particular metastasis to the liver.[33]

Inflammatory diseases have also been associated with elevated TP levels. Part of the increase in TP levels with inflammation might be due to the high level of TP in macrophages. Another explanation is that inflammatory cytokines, such as IL-8, tumor necrosis factor-α, and interferon-γ, upregulate TP.[34-36] Rheumatoid arthritis, inflammatory bowel disease, and psoriasis are all associated with an increase in TP, which may also play a part in the atherosclerosis process.[19,36-38]

1.5.1 TP and angiogenesis

The process of angiogenesis involves several different steps, including degradation of the preexisting extracellular matrix, proliferation of endothelial cells, migration, and tube formation. Neoangiogenesis, which is

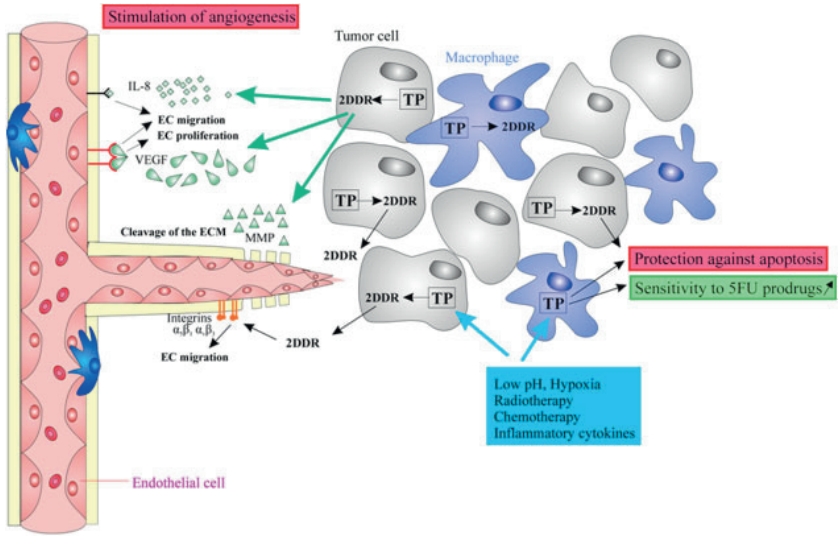

Figure 4. *Thymidine phosphorylase (TP) is found in tumor cells, as well as in macrophages. TP and its metabolite 2-deoxy-D-ribose-1-phosphate (in the picture given as 2DDR, otherwise 2-DDR-1P) stimulate angiogenesis by inducing endothelial cell migration and the secretion and/or expression of angiogenic molecules. TP and 2-DDR-1P may also provide resistance to apoptosis induced by hypoxia, and they are involved in the metabolism of 5-FU and its prodrug capecitabine. Reprinted by permission from John Wiley and Sons.*

important for tumor evolvement, is a process that depends on a good blood supply, which is also important for a tumor's ability to metastasize. TP and its catalytic product 2-DDR-1P are involved in the process of the formation of new blood vessels through stimulation of endothelial cell migration and tube formation (Figure 4).[20,23,39,40] TP has been associated with increased microvessel density in tumors, and it is known to be associated with VEGF, another angiogenesis factor.[39-41] Other studies have shown that a high vessel count is associated with high TP levels when VEGF is low.[27] In hypoxic environments, TP and 2-DDR-1P may also provide resistance to apoptosis.[19,42]

1.5.2 TP in relation to chemo- and radiotherapy

TP is involved in the metabolism of 5-FU, and it catalyzes the final step of the oral prodrug capecitabine to 5-FU (Figure 5). Because TP levels have been shown to be higher in tumors compared to normal tissues, it has been suggested that a preferential activation of capecitabine occurs in tumor tissue.

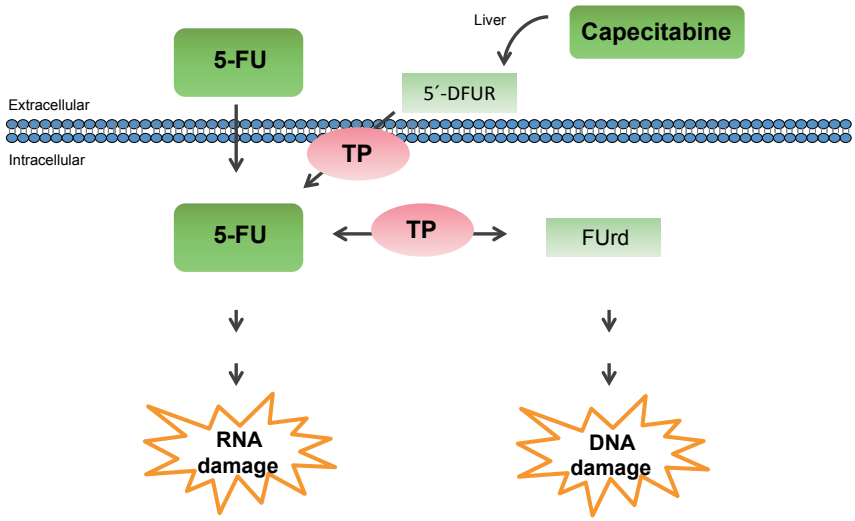

Figure 5. *Thymidine phosphorylase (TP) catalyzes the final and rate-limiting step of capecitabine conversion to 5-FU. One of the activation pathways of 5-FU is the conversion of 5-FU by TP to FUrd, which, through several steps, results in an inhibition of DNA synthesis.*

Although several studies support this theory, other studies have reported contradictory results. [22,43-47] Some have reported an association between resistance to 5-FU chemotherapy and high TP expression.[3,48,49] In contrast, it has been shown that cells transfected with TP cDNA have a higher sensitivity to 5-FU compared with control cells.[50-52] No correlation between TP and 5-FU response has also been reported.[53] Studies on cell lines and human cancer xenografts have indicated that TP levels increase after irradiation (Figure 4).[54,55]

2 AIM

The aims of this thesis were to:

- Evaluate the correlation between TP gene expression and known prognostic factors in CRC, and to evaluate whether TP has a predictive value for chemotherapy-induced side effects.
- Determine whether radiotherapy imposes a change in TP gene expression in rectal cancer tumors.
- Determine whether TP gene expression in tumors or mucosa has a predictive value for chemotherapy response in CRC.
- Evaluate whether there is a correlation between serum TP and TP gene expression in tumor tissue.
- Evaluate the adherence to practice guidelines for adjuvant chemotherapy for CRC in Sweden.

3 PATIENTS

A summary of the included patient cohorts is presented in Table 2.

Table 2. *Overview*

Paper	Patient cohorts	Number of patients	Location	Inclusion period
I	Stage III colorectal cancer	254	SU/Östra	2001–2009
II	Stage I–III rectal cancer	28	SU/Östra	2006–2008
III	Advanced colorectal cancer	125	SU/Östra	2002–2011
IV	Stage II–III colorectal cancer	17,521	Sweden	2007–2012

3.1 Paper I

Patients who were treated with adjuvant 5-FU-based chemotherapy and underwent resection surgery for stage III CRC at Sahlgrenska University Hospital/Östra (SU/Östra) from November 2001 until August 2009 were included in this study. During the study period, 375 patients fulfilled the inclusion criteria, and tissue samples were available from 254 of these patients.

3.2 Paper II

The patient cohort for this study was linked to a study of neoadjuvant chemotherapy use in rectal cancer.[56] That study was conducted from June 2006 to January 2008 and included 37 patients. The biopsy serie of three were completed in 28 of the patients, who were included in this study.

3.3 Paper III

Patients included in this study were treated with 5-FU-based first-line chemotherapy for stage IV CRC after undergoing resection surgery for CRC between 2002 and 2011 at SU/Östra. Biopsies were available from 125 patients, and matched serum samples were available from 70 of those patients.

3.4 Paper IV

This study included national data from the Swedish Colorectal Cancer Registry (SCRCR) of patients diagnosed from 2007 through 2012. Of almost 34,000 patients registered, 17,521 were in stage II or III and were included in the final studied cohort.

4 METHODOLOGICAL CONSIDERATIONS

A summary of the study designs, methods, and statistical analyses of the four papers is presented in Table 3.

Table 3. *Overview of methods*

Paper	Study design	Methods	Statistics
I	Experimental study	Quantitative real-time PCR	t-test/ANOVA Chi-square/Kruskal–Wallis Pearson correlation coefficient (r)
II	Experimental study	Quantitative real-time PCR	Descriptive t-test/ANOVA Matched-pair analysis
III	Experimental study	Quantitative real-time PCR ELISA	Descriptive Chi-square/Kruskal–Wallis Pearson correlation coefficient (r) Kaplan–Meier survival Log-rank test Logistic regression Cox proportional hazard
IV	Cohort study	Epidemiological analysis of registry data	Descriptive Logistic regression Multivariate logistic regression Wald tests

4.1 The database and biobank (Papers I-III)

In Gothenburg, most colorectal operations are performed at SU/Östra. A local CRC registry established on a continuous basis in 1999 is rather complete. Clinical data, information from the pathology report, and chemotherapy and

radiotherapy results are gathered from the medical records. Medical records are reviewed on a yearly basis.

A biobank (No 242) was started at the same time as the database. Tumor tissue and normal-appearing mucosal tissue samples are collected from patients undergoing resection surgery for CRC. The fresh samples are then snap-frozen in liquid nitrogen. In recent years, blood and saliva samples have also been gathered for the biobank.

4.1.1 Validation and limitations

A biobank connected to a continuously updated clinical database generates a multitude of information for researchers. However, some patients may be underrepresented in this biobank; for instance, the samples are collected during office hours. Patients who are operated on during off-hours might be more likely to represent the high-risk criteria of clinical presentation, with intestinal occlusion or perforation. Depending on the type of surgery and the difficulty of the dissection of the tumor, the time of ischemia during surgery may vary. The time from ligation of the large vessels of the tumor until the tissue sample is put in the freezer is an influencing factor. The inflammatory response during surgery also might affect the results, depending on what is being analyzed.

4.2 Quantitative Real-time PCR (Papers I-III)

Real-time PCR is a method that can be used for gene expression analysis and single-nucleotide polymorphism analysis, as well as for detecting chromosome aberrations.[57,58] Quantitative real-time PCR is used to analyze the actively transcribed genes; i.e., the gene expression level in cells or tissue. The sequence of the boundary regions of the DNA to be amplified must be known. To analyze gene expression in tumor or mucosa, total RNA is isolated from the tissue and purified. Because RNA is single-stranded and sensitive to degradation, the RNA is converted to the more stable cDNA, in a process called reverse transcription. Then, primers and a fluorescent probe corresponding to the target sequence are added to the cDNA sample. To ensure that there is no contamination, a negative control is included in each run. The real-time PCR also needs a fluorescent dye for reporting fluorescence. During the PCR amplification, which is a thermal cycling process, the amount of the target DNA sequence is doubled in each cycle. To quantify the gene expression, a threshold value is set sufficiently above

background, and the number of cycles required to reach the threshold is called the Ct value. To calculate the relative gene expression, an endogenous control is used as a reference gene to normalize for input RNA levels and the efficiency of the reverse transcription reaction. A schematic overview of the quantitative real-time PCR process is presented in Figure 6. Primers and probes used in papers I–III are listed in Table 4.

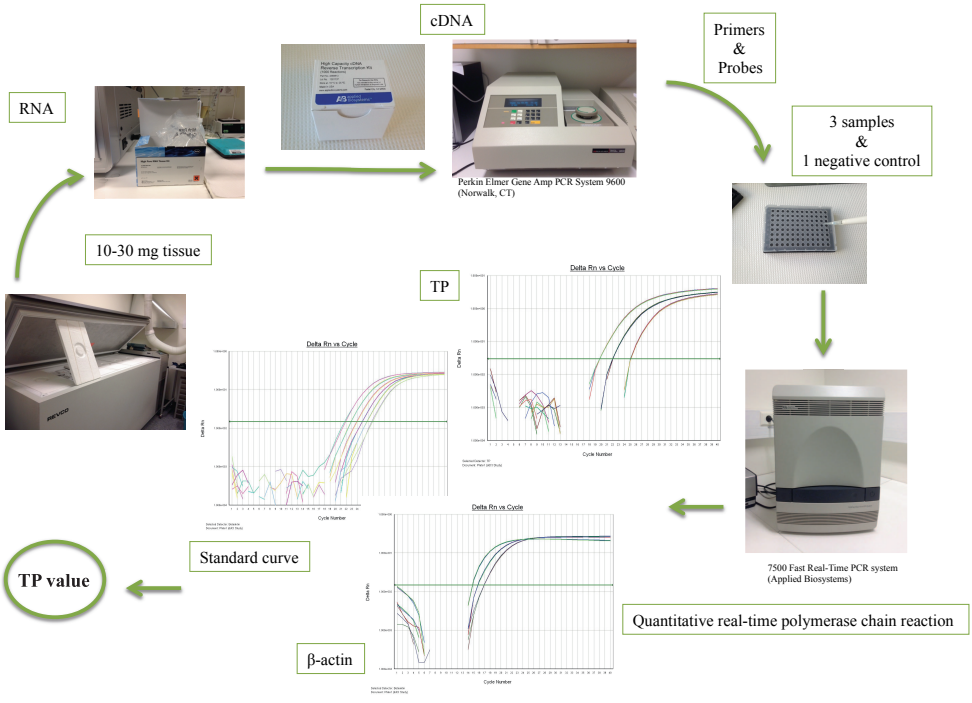

Figure 6. *From tissue to a thymidine phosphorylase (TP) value. First, RNA was isolated from 10–30 mg snap-frozen tissue. Then, cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time quantitative PCR was performed using the 7500 Fast Real-Time PCR system (Applied Biosystems). TP transcript levels were quantified using Assays-on-Demand™ from Applied Biosystems (Hs00157317_m1). β-actin was used as an endogenous control to normalize for RNA levels and the efficiency of the reverse-transcription reaction.*

Table 4. *Primers and probes used in the real-time-polymerase chain reaction for thymidine phosphorylase (TP) and the endogenous control β-actin*

TP	Probe	5′-CAG CCA GAG ATG TGA CAG CCA CCG T-3′
TP	Forward primer	5′-CCT GCG GAC GGA ATC CTA TA-3′
TP	Reverse primer	5′-TGT GAT GAG TGG CAG GCT GT-3′
β-actin	Probe	5′-CCT GAA CCC CAA GGC CAA CCG-3′
β-actin	Forward primer	5′-CGT GCT GCT GAC CGA GG-3′
β-actin	Reverse primer	5′-GAA GGT CTC AAA CAT GAT CTG GGT-3′

4.2.1 Validation and limitations

To minimize operator variability, a single operator performed the tissue preparations and analyses. The same amounts of tissue, and the same assay, and reference gene were used throughout the studies, making the internal validity rather good. However, there are some issues with the method. When analyzing the gene expression, it is the actively transcribed genes, but we do not know for certain that all of those mRNAs would be translated into actively working proteins. One small study has shown a correlation between TP gene expression and TP enzyme activity in CRC, which does support the connection between gene expression and actively working proteins.[24] Factors associated with time of ischemia and time until the biopsies are snap-frozen (page 18) might affect the gene expression, as mRNA is fragile and readily starts to degrade. Whether the biopsy is frozen or the analyses are performed on paraffin-embedded material are other factors that can potentially affect mRNA levels. Long-term storage of the samples further adds to RNA degradation caused by formalin fixation. Despite these issues, these fragmented transcripts might still be able to be reliably quantified by real-time PCR, provided that short amplicons and proper normalization genes are used.[59] Ischemia and handling of the biopsies are factors that are expected to affect TP and the reference gene equally. The calculation of a delta CT value between the target gene and one or several housekeeping genes compensates for the degradation of RNA. Delta CT are less sensitive to the fragmentation

of RNA caused by ischemia or handling of the tissue and are unaffected by varying amounts of input RNA.[60]

4.3 Enzyme-linked immunosorbent assay (Paper III)

Enzyme-linked immunosorbent assay (ELISA) is a way of detecting and quantifying protein. By using an antibody–antigen reaction and an enzyme reaction, this technique usually converts a peroxidase-sensitive substrate into a color (Figure 7). Absorption at a specific wavelength is quantified by a spectrophotometer to assess the concentration. Internal standards of known concentrations are used to quantify the optical densities of the test sample.

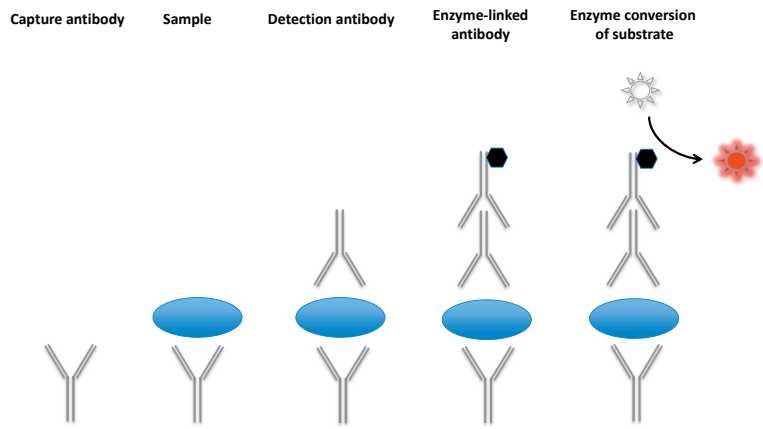

Figure 7.*Enzyme-linked immunosorbent assay (ELISA). First, a plate is coated with a captured antibody, after which the sample (and standard solution) is added and the antigen binds. A detection antibody binds to the antigen, and a secondary enzyme-linked antibody is added and binds to the detection antibody. A substrate is added and converted by the enzyme to a detectable form, such as red color, and then quantified by a spectrophotometer to assess the concentration of the sample. The plate is incubated and washed between each step.*

4.3.1 Validation and limitations

Intra-assay variations are variations among several samples within the same plate, and inter-assay variations are variations among several plates. The

intra-assay variation in paper III was <10%, and the inter-assay variation stated by the manufacturer was 12%. A single operator, excluding intra-operator variability, performed all protein extractions and ELISA assays.

For quality control of the assays and to evaluate inter-assay variability, each plate included samples with known concentrations and a reagent blank. To standardize the results, the concentrations were normalized to the total protein content of each sample.[61] Unexpected values that differed more than 10% among the replicates were reanalyzed.

Although the antibody–antigen reaction in the initial step makes these assays very specific to the target, depending on what epitope the antibody is directed against, the assays cannot always discriminate different conformations of a protein, such as latent or active form.

4.4 The Swedish Colorectal Cancer Registry (SCRCR) (Paper IV)

The first part of the SCRCR was launched in 1995 and included all rectal adenocarcinomas diagnosed in Sweden. Since 2007, colonic adenocarcinomas have been included as well. Other tumors (e.g., sarcoma, squamous cell carcinoma, and overgrowth from adenocarcinoma in other organs) are not included. Today, a mandatory cancer notification goes directly to the registry at the time of diagnosis. Tumors identified at autopsy are not included in the registry.

The main purposes of the registry are to audit management and outcome, report data for quality improvements, and provide valid data for research. The registry has been validated against medical records for a full-year cohort and has shown 94–97% agreement on six variables; a study on the registry's first three years deemed the validity as "good."[62] A structured validation is currently being performed on all variables in 20% of patients diagnosed during one year.

Quality registry data includes preoperative parameters, such as doctors' assessments and whether a CT scan and MRI were performed; operative information, such as which surgery was performed and the level at which the surgical tie was made; and postoperative parameters, such as preoperative and postoperative MDT conferences and planned chemotherapy and

radiotherapy. In 2009, this quality registry data was extended. It now also includes an oncology form in which the department responsible for the oncological treatment reports data on chemotherapy and radiotherapy results.

4.4.1 Validation and limitations

The large patient cohort in this registry-based study is an advantage, and the coverage is more than 99% of all diagnosed adenocarcinomas in Sweden, making this a truly population-based registry.[9,63] However, the large number of patients also makes the importance of validity stand out. Part of the registry was validated at the beginning of the registry, and there is no reason to believe this has changed. However, the validity of different parameters within the registry differ; for instance, we know that information on vascular or perineural invasion are often lacking in the pathology report, and lymphatic invasion is not even reported in the SCRCR.

Another problem in registry research is the large cohort number that is needed in order to be able to rely on the registry data; it is not possible to check a medical record if information is lacking. For instance, in paper IV, the information about the cancer stage was missing in several cases, even though resection surgery had been carried out. Reasons for missing the cancer stage could be that the box regarding metastasis was not checked. The SCRCR is a living registry, and information changes over time. The date of the data was obtained is important, as one cannot go back and retrieve extra information if something was missed. Yet another problem related to this type of research is the change in parameters included in the registry over time.

4.5 Statistical considerations

Descriptive statistics of demographics are shown using contingency tables. Values are presented as median (range) or means (standard deviation).

Statistical comparisons were made mainly by t-test/ANOVA, but due to the limited number of patients, they were also crosschecked by non-parametric tests. Chi-square or Kruskal–Wallis tests were used for non-parametric testing. Treatment-related changes were also assessed by a non-parametric test in matched-pair analysis. The Pearson correlation coefficient (r) was used

to compare sets of continuous parameters. The significance level set throughout the studies was 95%.

Univariate logistic regression was applied to assess the putative relation of classical risk factors on the outcome, quantified in terms of 95% confidence intervals. In order to adjust for possible confounding, the factors of interest were included in a multivariate logistic regression analysis. Confidence intervals and Wald tests were used to evaluate significance in the multivariate analyses.

Survival and time-to-event curves were calculated by the Kaplan–Meier method, and statistically significant differences in survival were calculated using the log-rank test. Relative risk was assessed by the univariate and multivariate Cox proportional hazard models.

The software used for calculations in paper I and II was the JMP 8.0/SAS software program (SAS Inc., Cary, NC). In paper III, the JMP 10.0 (SAS Inc., Cary, NC, USA) was used, and in paper IV, the R 2.15.1 software (R Core Team, 2014)[64].

4.6 Ethical considerations

All of the studies were performed with full consideration of and adherence to good clinical practice. As part of the clinical routine and with the aid of our study nurses, the patients were informed of the projects, the collecting of material, and the aim and goal of their use. Acceptance of participation was documented, and all patients had the opportunity to decline participation. Papers I, II, and III: The Regional Ethical Review Board of Gothenburg gave its approval before the database was set and before gathering of the material began (Ö445-00). Paper IV: The Regional Ethical Review Board of Gothenburg approved the study (Dnr 073-13).

5 RESULTS

5.1 Paper I

The relative TP gene expression in the tumor samples (tTP) did not relate to age or differ between genders. Mucosa samples, however, did show a negative correlation with age (r= –0.14, p=0.026). There was no correlation between tTP and mucosa TP (mTP) gene expression.

The tTP gene expression correlated to the number of lymph nodes positive for tumor cells (r=0.15, p<0.05), and the mean value was higher with a worse N-stage (p<0.05), as presented in Figure 8. Likewise, tTP gene expression increased with worse tumor differentiation (p<0.05).

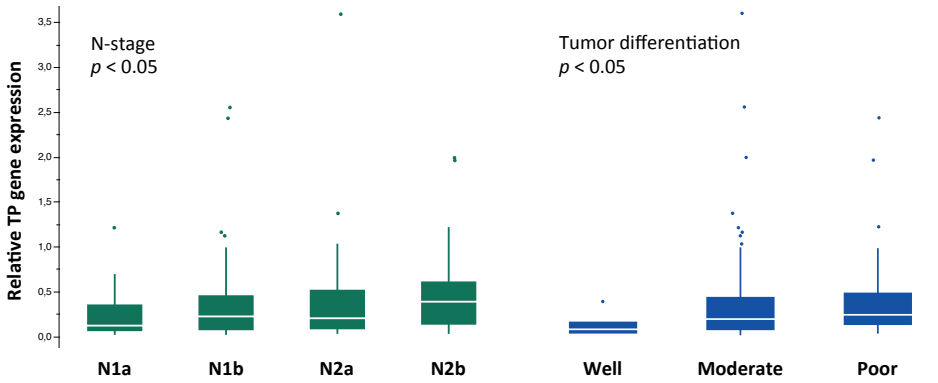

Figure 8. *Thymidine phosphorylase (TP) gene expression in colorectal tumors stage III by N-stage and tumor differentiation. The distribution of TP expression is displayed as box-and-whisker plots with median, 25%, and 75% quartiles (boxes), minimum and maximum (whisker), and outliers (dots). Abbreviation: N=node*

In patients with rectal cancer, there was a difference in mTP gene expression between patients who received radiotherapy preoperatively (n=40, 0.40 ± 0.26) and those who did not (n=75, 0.30 ± 0.22) (p<0.05). This difference was not seen in the corresponding tumor samples. However, the mean tTP was higher in rectal tumors compared to colon tumors (0.46±0.54 and 0.27±0.29, respectively, p<0.05).

No association was found between mTP or tTP gene expression and a specific chemotherapy-related side effect, but there was a higher risk of need for dose reduction with higher tTP (p<0.05). High mTP values were

associated with preemptive discontinuation of adjuvant chemotherapy due to toxicity (p<0.05).

5.2 Paper II

The timing of biopsies and study overview are presented in Figure 9.

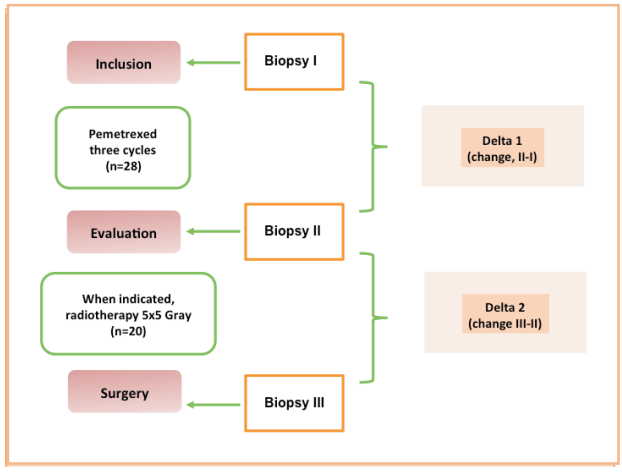

Figure 9. *Overview of the pemetrexed study, showing the time of biopsy sampling. Alterations in thymidine phosphorylase (TP) gene expression were expressed as delta values.*

Of 28 patients receiving chemotherapy, 20 patients also received preoperative radiotherapy. There was an increase in tumor TP gene expression after radiotherapy (p<0.05, see Figure 10).

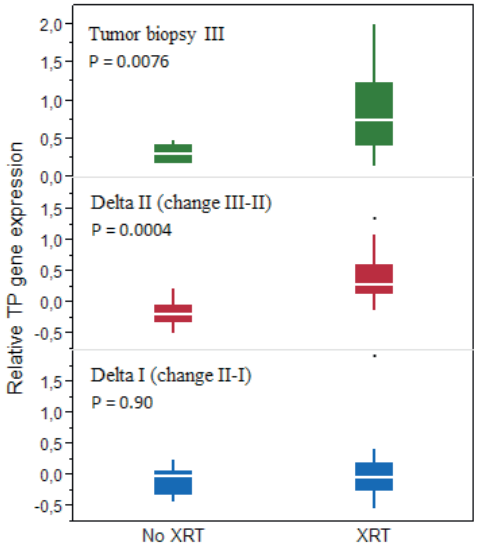

Figure 10. *Box plots showing an increase in relative thymidine phosphorylase (TP) gene expression in rectal tumors after radiotherapy (5x5 Gy). Tissue was obtained before treatment (biopsy I), after completion of chemotherapy (before radiotherapy, if given; biopsy II), and at the time of surgery (biopsy III). The distribution of TP expression is displayed as box-and-whisker plots with median, 25% and 75% quartiles (boxes), minimum and maximum (whisker), and outliers (dots). An elevation in TP gene expression was seen after radiotherapy and not after chemotherapy alone.*

In patients not eligible for radiotherapy, there was a significant decrease in tTP between biopsies I and III (p<0.05; see Figure 11).

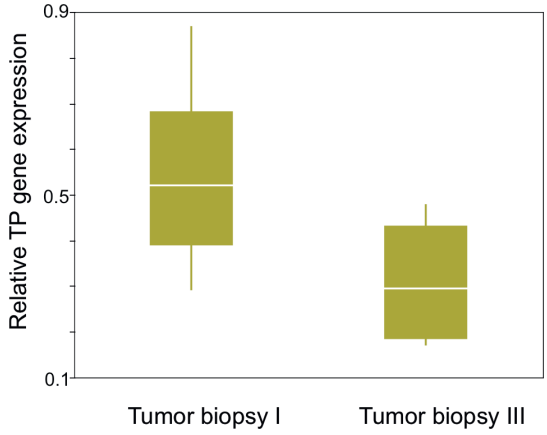

Figure 11. *Box plots showing a decrease in relative thymidine phosphorylase (TP) gene expression in rectal tumors after chemotherapy. Tissues were obtained before treatment (biopsy I) and at the time of surgery (biopsy III). The distribution of TP expression is displayed as box-and-whisker plots with median, 25%, and 75% quartiles (boxes), and minimum and maximum (whisker).*

5.3 Paper III

The TP gene expression of matched tumor and mucosa tissues showed a positive correlation (r=0.41, $p<0.05$). However, no correlation between TP gene expression in the tumor (n=125) and matched serum (n=70) samples was seen. High tTP gene expression was associated with a longer time to progression ($p<0.05$; Figure 12, left panel). However, there was no association with the response rate of the tumor or with palliative survival (Figure 12, right panel). When tTP was included in a multivariate analysis that also included gender, age, tumor location, differentiation, Eastern Cooperative Oncology Group (ECOG) performance status, and single or combination chemotherapy treatment, tTP ($p<0.05$) and ECOG ($p<0.05$) were the two factors associated with time to progression.

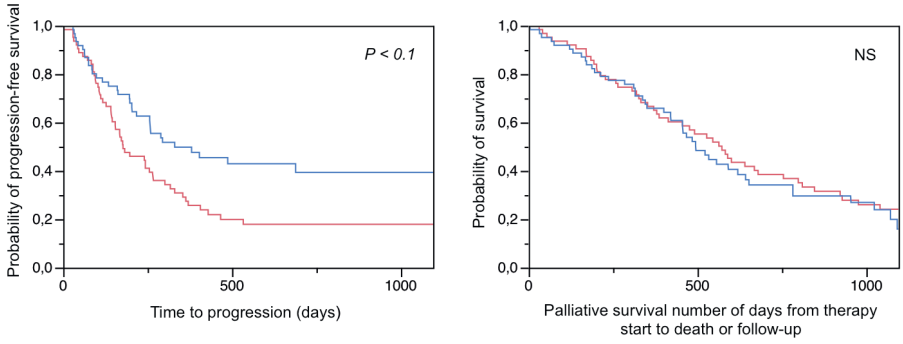

Figure 12. *Left panel: Patients with high thymidine phosphorylase* (TP) *gene expression* (*n=62, blue line) in tumor tissues exhibited significantly longer time to progression compared with patients with low expression (n=63, red line). Right panel: There was no significant difference in palliative survival rate according to high and low TP gene expression.*

There was no association between mTP and tumor response or time-to-event variables.

5.4 Paper IV

Two slightly different patient cohorts were analyzed in this study. The first, was patients registered in the SCRCR (2007–2012), and the primary outcome of interest was *planned adjuvant chemotherapy* in stage II–III CRC. The second, was patients for whom additional information on chemotherapy and

radiotherapy (2009–2012) were available, and the outcome of interest was *started adjuvant chemotherapy.*

SCRCR dataset (2007-2012)

Colon cancer stage III

The Swedish and international guidelines recommend adjuvant chemotherapy for patients with stage III colon cancer.[13,14,16,65] Factors possibly influencing whether patients were planned for adjuvant chemotherapy were included in a multivariate analysis, which showed that factors associated with planned adjuvant chemotherapy were age, gender, comorbidity, N-stage, and MDT conference (Figure 13).

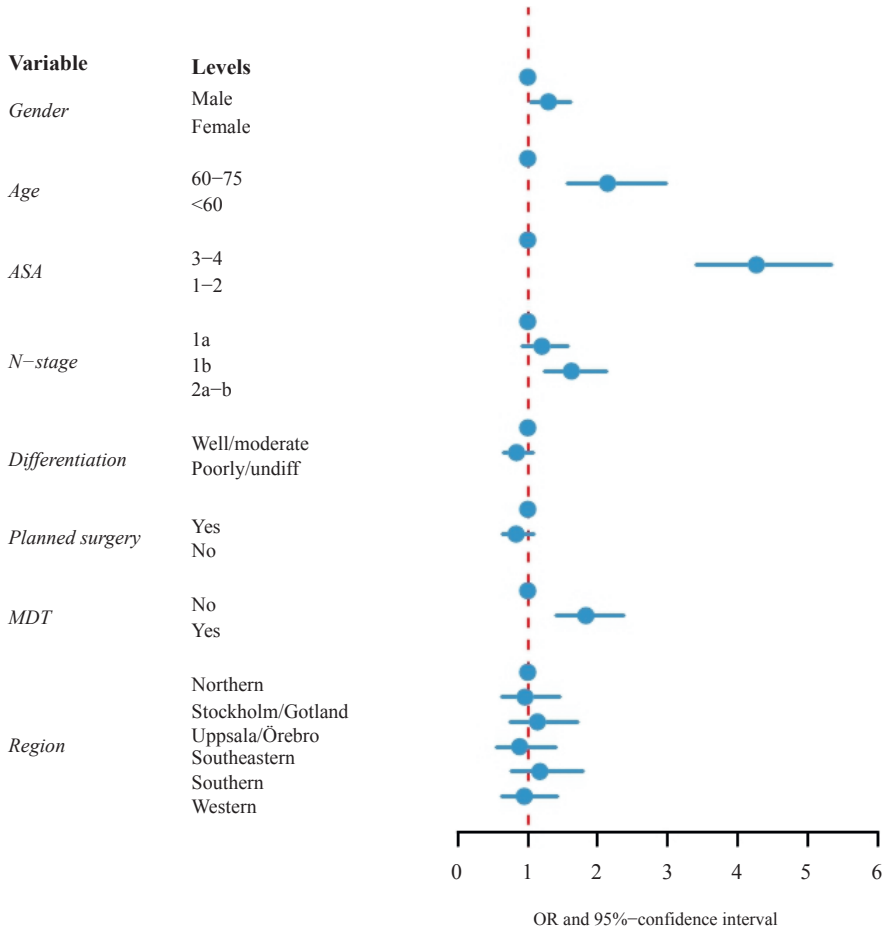

Figure 13. *Patients planned for adjuvant chemotherapy for colon cancer stage III by patient and healthcare region (n=3427). Complete case multivariate logistic regression.*

Colon cancer stage II

Patients with stage II colon cancer were divided into two groups — one with low risk of recurrence and one with high risk of recurrence. The Swedish guidelines state that patients with high-risk stage II colon cancer might be considered for adjuvant chemotherapy as if they were stage III. High-risk criteria are emergency intestinal occlusion or perforation, lymph node sampling less than 12, T4 tumor, poorly differentiated tumor, and vascular or perineural invasion. Less than half of the patients younger than 76 years of age with one or more high-risk criteria were planned for adjuvant chemotherapy. There was an increase in patients receiving adjuvant chemotherapy over time.

Rectal cancer stages II–III

The current Swedish guidelines do not recommend adjuvant chemotherapy to treat rectal cancer; however, international guidelines do, even though there is still no clear consensus due to less scientific evidence compared to colon cancer.[13,15,17,66] Compared to colon cancer, the proportion of patients planned for adjuvant chemotherapy was lower, although there was an increase over time.

Oncology dataset (2009–2012)

Adjuvant chemotherapy was started more often in colon cancer than in rectal cancer groups. Combination chemotherapy with capecitabine/5-FU and oxaliplatin was prescribed more often for stage III N2 than for stage III N1 and stage II. In both colon and rectal cancer groups, one-third of the patients started adjuvant chemotherapy more than eight weeks after surgery.

6 GENERAL DISCUSSION AND COMMENTS

6.1 Papers I–III

It is important to find prognostic and predictive biomarkers for CRC and chemotherapy treatment. TP is an enzyme with several tumor-promoting properties (see introduction), and the enzyme is involved in the activation of 5-FU and its prodrug capecitabine, making it interesting for further evaluation. Several studies have been conducted on TP in CRC, but with divergent results. One of the reasons behind the contrasting results might be the selection of patients with different demographic and clinicopathological backgrounds; in addition, the methods of analyzing TP may generate inconsistent results.

In paper I, an association was found between TP and known prognostic factors in CRC. A positive correlation was seen between TP and the number of positive lymph nodes. The results also showed that high TP levels were connected to worse N-stage and less differentiated tumors. A clinical implication of these results might be to measure the TP level in a preoperative biopsy. TP levels measured preoperatively might add information to the preoperative staging that becomes important when neoadjuvant chemotherapy is considered.

In paper III, the predictive value of TP was supported by the results. High TP gene expression was related to a longer time to progression related to the effect of treatment. On the other hand, no significant relationship between the gene expression level of TP and tumor response or palliative survival was found.

Rectal tumors had higher TP gene expression levels compared with colon tumors (papers I and III). We hypothesized that there was a connection to radiotherapy, although there was no significant difference in TP expression between rectal tumor tissues when subgrouped according to whether or not radiotherapy was administered (paper I). In paper II, we were able to analyze the change in relative TP gene expression before and after radiotherapy, and the results showed an increase in TP levels after irradiation, which supported this theory. Radiotherapy has been associated with a change in biomarkers

through inflammation and matrix remodeling.[67,68] Thus, it is plausible that radiotherapy induced an increase in TP as part of the inflammatory process. In addition to the increase in TP seen after radiotherapy, there was a slight decrease in TP in patients treated with chemotherapy alone. This result is supported by a few other studies.[69,70] TP has a key role in the activation of the chemotherapeutic agent capecitabine, which is often used preoperatively in combination with radiotherapy in rectal cancer. In a small study, upregulated TP was associated with a better response to chemoradiation.[71] One strength of our study was that each patient served as his or her own control, with three consecutive biopsies from the same tumor. However, the number of patients was limited; the selection of patients was not prospective for this specific study, and the stages varied. Furthermore, the chemotherapy pemetrexed is not the standard treatment, and it might influence TP value. However, because the change and not the absolute value of TP was analyzed in the same tumor before and after the given therapy, these factors should not have an impact on the results.

In paper I, the analysis of TP as a predictive marker for toxicity was difficult to evaluate, as there were no significant correlation with any specific side effect. Instead, TP correlated more with a general need for dose reduction and preemptive termination of treatment. The dose reduction was linked to tumor TP but not to mucosa TP, and the preemptive termination was linked to mucosa TP, but not to tTP. These two results go in different directions, and further studies are needed to clarify whether there is a connection between TP and toxicity with 5-FU treatment. It could be hypothesized that the TP level in the mucosa would be of most interest for a systemic or gastrointestinal side effect, as the tumor itself is comparably small.

In paper III, we also analyzed whether serum TP protein expression correlated with tumor protein expression. Other groups have reported that plasma or serum TP levels are elevated in cancer patients.[30,31] Associations with prognosis and predictive value have also been reported.[30-33] Our results showed that there was no association between TP gene expression in tumors or mucosa and serum TP protein level, tumor response, or time-to-event variables. However, there are several limitations to this part of the study. Serum samples were not available from all patients, and comparison analyses were conducted on 70 out of 125 patients. Therefore, selection bias might be present, as there were no patient serum samples for the first couple of years of the time period in this study. In addition, we do not know how fast serum TP changes in response to environmental factors. The exact timing of blood sampling was not known, and even the induction of anesthesia might change the level. To evaluate the potential predictive value of TP, a larger patient

cohort and repeated measurements of serum TP before and during treatment would be necessary.

In paper I, which related TP to known prognostic markers, there might be selection bias in the patient cohort, as tissue samples were only collected during office hours, and patients with high-risk factors such as perforation might be underrepresented. The same selection, of course, might be present in paper III, but it does not affect the results in the same way, as all patients in this group developed advanced disease. Instead, paper III has the problem of heterogeneity of the patient cohort. Initially, the included patients were in several different stages, and this difference does affect the type of treatment the patients received before the initiation of first-line treatment.

Other limitations are that the biopsies from tumors were collected during primary surgery, and the time span from surgery until the patient received first-line chemotherapy could vary from a couple of months to several years. However, there are studies supporting the value of analyzing gene expression in a primary tumor and relating it to the metastatic disease.[49,72,73]

One of the inclusion criteria (papers I and III) was 5-FU-based chemotherapy, as it is the chemotherapy associated with TP (see introduction). 5-FU-based chemotherapy is 5-FU in combination with leucovorin, but it also includes capecitabine. Capecitabine is dependent on TP in its last conversion step to its active metabolite, 5-FU. A preferred activation in tumor cells due to the higher level in tumors compared to normal tissue has been reported.[22,43-45] In our material (papers I and III), the patients mainly received 5-FU, and only few patients received capecitabine. Previous studies, including one from our group, associated high TP expression with a resistance to 5-FU.[3,48,49] With this in mind, the results of paper III were a bit of a surprise. The discrepancy, at least in part, is probably related to methodological issues. In this study, as well as in papers I and II, real-time PCR was performed on non-microdissected tumor tissue, which probably also included the TAMs. TP is predominantly expressed in the macrophages and they play an important role in the tumor microenvironment.[40,74-76]

Many studies on TP involve immunohistochemistry, in which researchers have the advantage of seeing what they are analyzing. However, the method is investigator-dependent, and the classification (color intensity, number of cells, and sometimes, positive/negative) and subgroups can differ between studies and make them difficult to compare. Tumors are heterogeneous, and protein and gene expression vary among the different locations of the tumor

(Figure 14). The cells that exhibit the highest levels of expression can also differ among tumors. Relative TP gene expression was analyzed in macroscopically dissected tumor tissue, including all types of cells, generating a larger portion of tumor tissue that was analyzed compared with microdissected tumor samples that mostly included tumor epithelial cells. The rationale behind this method was that we also wanted to include the stroma in the analysis of TP.

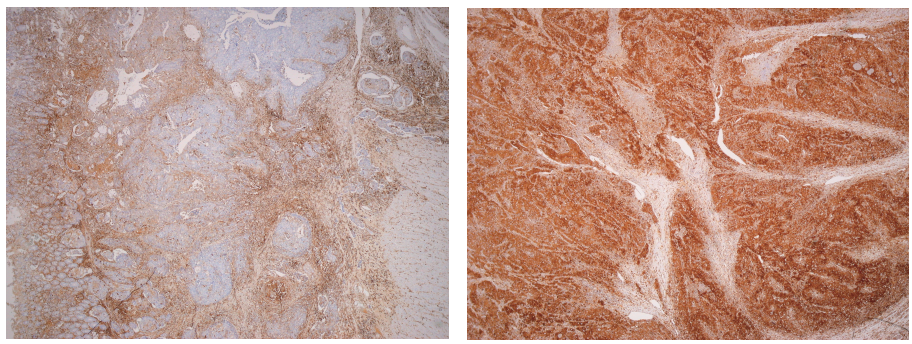

Figure 14. *Immunohistochemistry illustrating the heterogeneity of TP protein expression (brown) in and between two different colorectal tumor samples.*

Another limitation of paper I might be the lack of survival measures, such as overall survival. In the paper, we described a correlation between TP gene expression and some of the best-established prognostic markers in current use. When analyzing survival, it is common to divide patients into high/low expression groups to facilitate analysis. However, a cutoff then needs to be set, which usually is linked to the material at hand, thus making it difficult to apply the results to other studies and lowering the external validity. In paper I, we chose continuous TP value and opted not to assess and describe all possible survival measures. In paper III, however, the aim was to evaluate TP as a predictive marker for first-line 5-FU-based chemotherapy, making a dichotomy necessary; as such, we chose the median level as the cutoff.

6.2 Paper IV

There have been a few studies on adherence to guideline treatment, and the results mostly point toward a stage- and age-dependent difference in

adherence.[77-83] This study does support those findings and presents good adherence to clinical guidelines in younger and healthier patients.

However, there were subgroups with lower adherence to guidelines. The multivariate independent factors associated with planned adjuvant chemotherapy were age, comorbidity, N-stage, and MDT conference (Figure 13). Healthcare regions did not have an impact in this analysis, which seems to be true in the large picture. However, when subdividing further, some differences emerge; for example, the proportion of patients receiving a combination treatment is associated with better survival, although there are more side effects.[17,84-86] Results can be affected by how the patient cohort is subgrouped and the level on which the analysis is performed. However, this study was on guideline adherence, and both single and combination therapy were included within the guideline treatments.

In managing such a large cohort, the need for a statistician became obvious. It is important to know which patients are included and to be aware of shortfalls. Then, the difficult challenge is for researchers to establish causality between their findings.

There are limitations to a study based on registry data. Although this was a large, population-based patient cohort and the data were from a nationwide registry, several patients were lost in the analysis due to missing data. In addition, with a registry-based study, it is not possible to go back and check medical records for the information. The SCRCR is a living registry, with information and number of patients that is constantly changing; thus, it is not possible to go back and extract additional information on one parameter. The data are not consistently validated, and information bias is a possibility. However, for parameters with no obvious major shortfall, the missing data is probably random, and as the patient cohort is rather large, it probably does not affect the results. The data registration is prospective, and there are automatic warnings regarding the registration of extreme values or illogical combinations. Issues that arose during our data analysis were the evolvement and change of definitions over the time span of six years. For instance, the TNM staging system (see introduction) has become more detailed, and the registry did not include all detailed information on subgroups. For example, patients who would now be in stage III because of N1c, are considered stage II in our material.

Most of the analyses were carried out on patients who were planned for chemotherapy, and some on patients who started chemotherapy. While the registry does not include how many chemotherapy cycles the patients

actually did complete, time to start of chemotherapy was evaluable, and one-third of the patients initiated their treatment more than eight weeks after surgery. Studies have shown that starting adjuvant chemotherapy early is preferable, and trials that have shown a significant survival gain with adjuvant chemotherapy in colon cancer cases required an initiation of treatment within 5–6 weeks.[87-89]

Chemotherapy treatment for rectal cancer is difficult to evaluate. The Swedish guidelines currently do not recommend chemotherapy for rectal cancer; however, international guidelines recommend adjuvant chemotherapy for rectal cancer, even though there is a lack of evidence from randomized trials.[10,90-94] It has also been suggested that different approaches should be applied for rectal cancers located in the intraperitoneal and extraperitoneal part of the rectum respectively.[95] The inability to initiate adjuvant chemotherapy early enough in rectal compared with colon cancer patients has been suggested as one of the reasons it is harder to establish a clear benefit of adjuvant chemotherapy.[12]

7 CONCLUSIONS

- Higher intratumoral TP gene expression is related to worse N-stage and worse histological tumor grade. Thus, TP could be of potential interest as a prognostic marker, but further studies are needed in order to evaluate such use.

- Radiotherapy, and perhaps chemotherapy, affects the gene expression of TP.

- Serum protein expression of TP does not seem to correlate with tumor gene expression of TP.

- In non-microdissected tumor tissues of patients with advanced CRC, high TP expression is associated with a longer time to progression, which is related to the effect of treatment.

- The adherence to practice guidelines for adjuvant chemotherapy in CRC is rather good in younger and healthier patients. Special consideration should be given to patients over 60 years of age and with poorly differentiated tumors.

- MDT conferences affect whether or not a patient will be considered for adjuvant chemotherapy; this is true even in the absence of significant comorbidities.

- To obtain the best results from a given treatment, time from surgery until start of adjuvant chemotherapy needs to be reduced.

8 FUTURE PERSPECTIVES

A multimodal approach is needed to fight cancer. Finding tumors at an early stage is important. When a tumor has been established, the surgery should be performed meticulously. High-volume hospitals are probably best skilled when treating patients with tumors that are advanced.

Cancer is a heterogeneous disease, and treatment options are becoming more and more complex. Small tumors should just be resected, but preoperative staging is now of importance, especially in rectal cancer, for which there are several different approaches to give patients a better chance of achieving a cure. Due to the treatment options, MDT conferences are needed at several steps of the patient's journey through his or her disease. With today's technical solutions, this goal is possible, and it should not be very difficult to accomplish for all patients. Although the conferences are time consuming and involve several doctors, I do believe they give the patient the opportunity for the very best care.

The development of new techniques in genetic research has been rapid, and analyses performed in a short time span that can provide genome-wide data are already in use. As previously discussed, the complexity of carcinogenesis, from gene expression to final protein product, as well as the interaction of different signaling pathways affecting each other, make the results difficult to evaluate. Low expression of one important enzyme or gene can lead to upregulation of other, sending signals to a third party that will compensate for what was lost or inhibited in the first place. Numerous studies, many of apparently high-quality design, have been performed, but conflicting results are often presented. Collaborations among research groups to create larger sample sizes, more clearly defined patient cohorts, and growing biobanks will hopefully make future research move at a quicker pace. For instance, the EORTC is in the process of starting a biobank to analyze specific genes, and it will include patients from all over Europe. Perhaps in the future, when testing a new selective treatment that is only effective when used in patients with a specific or rather unusual gene mutation, researchers will be able to collaborate and include patients from all over Europe, resulting in a rather large patient cohort that otherwise would be impossible to achieve.

In paper I, there was a correlation between TP gene expression in CRC stage III and known prognostic factors. In paper III, a large proportion of patients with an initially good prognosis (stages I and II) was in the high TP group. It would be of interest to evaluate TP further in stage II to see whether TP could

add further information to other known prognostic risk factors. In addition, the connection to macrophages is of interest, and it would be interesting to evaluate further whether there is a difference in the expression of TP in TAMs and regularly differentiated macrophages. It could also be interesting to try to use TP elevation caused by irradiation to potentiate the effect of capecitabine in rectal cancer patients. We have pointed out that the method and the cells in which TP level is measured in the tumor might influence the study results. This issue needs to be addressed by comparing TP levels in the macrophages and tumor epithelial cells of macro- and microdissected tumors in different prognostic and predictive scenarios.

Finding new approaches in the battle against cancer is necessary to improve patient survival. By continuing the mapping of every step in the evolution of tumors, as well as their metastatic lesions, interactions between healthy and sick cells, the immune system, and the signaling pathways, new treatments could be generated, making it possible to tailor an individually optimized treatment plan for each and every patient.

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