

Linköping University Medical Dissertations

No. 1226

Biological and histological factors as predictors in rectal cancer patients – A study in a clinical trial of preoperative radiotherapy

Annica Holmqvist



Linköping University
FACULTY OF HEALTH SCIENCES

Division of Oncology
Department of Clinical and Experimental Medicine
Faculty of Health Sciences, SE-58185 Linköping, Sweden
Linköping 2011

Cover: Rectal tumour receives radiotherapy. Illustrated by Annica Holmqvist

© 2011 Annica Holmqvist

ISBN 978-91-7393-234-9

ISSN 0345-0082

Published articles have been reprinted with the permission of the copyright holders.

Paper I © 2004 Elsevier, International Journal of Radiation Oncology Biology Physics

Paper III © 2010 Oxford University Press, Annals of Oncology

Paper IV © 2006 Elsevier, Oncology Report

Printed by LiU-Tryck, Linköping, Sweden 2011

Till min älskade familj

Abstract

With improved surgical techniques and preoperative radiotherapy (RT) the local recurrence rate in rectal cancer patients has been reduced, however the mortality rate is still high and there is a huge variation in the response to preoperative RT in patients with the same tumour stage. To improve patient's survival, it is of great importance to identify good prognostic and predictive factors that help us to select the best suited patients for preoperative RT in the future.

For many years, studies of neoplastic transformation have mainly focused on tumour cells. In recent years, researchers have realised that the stroma around tumour cells and their extracellular matrix components also play an important role in tumour carcinogenesis.

The aim of this thesis was to investigate the biological factors, survivin and particularly interesting new cysteine-histidine rich protein (PINCH), histological factors, inflammatory infiltration, fibrosis, necrosis, mucinous content, angiogenesis and lymphangiogenesis as well as their relationships to preoperative RT and to clinical variables in rectal cancer patients who participated in a Swedish rectal cancer trial of preoperative RT.

In paper I, the expression of survivin and its relationship to preoperative RT and clinical factors were investigated in 98 primary rectal tumours and adjacent normal mucosa. In all patients, positive survivin expression was independently related to worse survival compared to negative survivin expression in a multivariate analysis.

In paper II, PINCH expression and its relationship to RT, clinical, histological and biological factors were investigated at the invasive margin and inner tumour area in 137 primary rectal tumours and in cell line of fibroblasts. In patients without RT, strong PINCH expression was independently related to worse survival in a multivariate analysis. No survival relationship was found in the patients with RT, and there was no difference in PINCH expression between the subgroups of non-RT and RT at the invasive margin/inner tumour area. In patients with RT, strong PINCH expression at the inner tumour area was related to a high level of lymphatic vessel density (LVD).

In paper III, the frequency of LVD/blood vessel density (BVD) was analysed at the periphery, the inner tumour area and the invasive margin of 138/140 primary rectal tumours and correlated to RT, clinical, histological and biological factors. In all patients, LVD at the periphery of the tumour was independently related to better survival compared to LVD at the inner tumour area/invasive margin. In all patients, a higher LVD at the periphery was related to negative (wild type) p53 expression.

In paper IV, the inflammatory infiltration, fibrosis, necrosis and mucinous content were studied in relation to RT, clinical and biological parameters in preoperative biopsies ($n = 153$) and in primary tumours ($n = 148$). In all patients and in the subgroups of non-RT and RT a higher grade of inflammatory infiltration was independently related to improved survival compared to weak inflammatory infiltration in a multivariate analysis.

In this thesis, survivin, PINCH, LVD and inflammatory infiltration are independent prognostic factors in rectal cancer patients who participated in a clinical trial of preoperative RT. This information may help us to improve patient's survival by selecting the best suited patients for preoperative RT in the future.

Table of contents

Abstract	5
Table of contents	7
Sammanfattning	9
Abbreviations	11
List of papers	13
Introduction	15
Background	17
Epidemiology	17
Aetiology and risk factors	17
Heredity	18
Pathology.....	19
Histological factors	22
Inflammatory infiltration.....	22
Fibrosis	24
Necrosis	24
Mucinous content	25
Angiogenesis	25
Lymphangiogenesis.....	26
Biological factors	27
Survivin	27
PINCH.....	29
Apoptosis.....	30
p53	30
Cox-2.....	31
Cell cycle.....	32
Treatment	33
Surgery	33
Radiotherapy	33
Chemotherapy and immunotherapy	35
Aims	37
Materials and Methods	38
Patients	38
Immunohistochemistry.....	40
Cell line analysis and radiation procedure	41
Western blotting	42
Haematoxylin and Eosin staining.....	42
Evaluation.....	42
Statistical analysis	43

Results	45
Paper I	45
Paper II	45
Paper III.....	46
Paper IV.....	47
Discussion	48
Conclusions	51
Acknowledgements	53
References	55

Sammanfattning

Strålbehandling innan operation och förbättrad operationsteknik har kraftigt minskat risken för lokalt återfall hos patienter med ändtarmscancer, trots detta så är dödligheten fortfarande hög. Vi har visat att proteinerna survivin, PINCH, antal lymfkärl och infiltration av inflammatoriska celler är starkt relaterat till överlevnad hos patienter med ändtarmscancer. Förhoppningsvis kan den här informationen hjälpa oss att förbättra överlevanden för patienter med ändtarmscancer genom att välja ut de patienter som har störst nytta av strålbehandling.

Tjocktarms- och ändtarmscancer är en av de vanligaste sjukdomarna i världen med över en miljon insjuknande varje år. I Sverige diagnostiseras cirka 5500 tjocktarms- och ändtarmscancerfall varje år, varav cirka 2000 är ändtarmscancer. I början av 1900-talet var utfallet av sjukdomen mycket dålig och 25-45 procent drabbades av lokala återfall. På 1980-talet visade forskare att strålbehandling innan operation i kombination med en ny operationsteknik kallad Total Mesorectal Excision, kunde minska risken för lokalt återfall till cirka fem procent. Trots den minskade risken för lokalt återfall så kommer cirka 40-50 procent av patienterna att avlida i sin sjukdom och det är fortfarande stora skillnader i effekten av strålbehandling hos patienter med tumörer av samma utseende, storlek och utbredning. Därför är det mycket viktigt att försöka hitta faktorer som kan hjälpa oss att förutsäga vilka patienter med ändtarmscancer som kommer att ha nytta av strålbehandling.

I flera år har forskare studerat tumörcellen och dess egenskaper i cancerutvecklingen, och de senaste åren har man sett att vävnaden runt omkring cancercellen också spelar en stor roll för utvecklingen av cancer.

I den här avhandlingen ville vi undersöka två proteiner i tumörcellen, survivin och particularly interesting new cysteine-histidine rich protein, PINCH, samt faktorer i vävnaden runt tumörcellen som mängden inflammatoriska celler, ärrvävnad (fibros), död vävnad (necros), slem (mucin), blodkärl och lymfkärl. Vidare ville vi undersöka hur dessa faktorer var relaterade till strålbehandling liksom till kön, ålder, tumörens aggressivitetsgrad och dess utbredning, patienternas överlevnad, risk för lokalt återfall och spridning av tumören till andra organ. Detta studerades hos ändtarmscancer patienter som deltog i en klinisk svensk studie där hälften av patienterna fick strålbehandling och hälften inte fick strålbehandling innan operation.

Tidigare studier har visat att survivin uttrycks i fostervävnad men inte i normal vävnad, halten av survivin ökar i tumörer och ett starkt uttryck av survivin har visat sig vara relaterat till en sämre överlevnad hos ändtarmscancer patienter som fått en kombination av strålbehandling och cytostatika. Vår studie var den första som analyserade förhållandet mellan survivin och enbart strålbehandling hos ändtarmscancer patienter. Vi kunde visa att patienter med survivin positiva tumörer hade en sämre överlevnad jämfört med patienter med survivin negativa tumörer.

PINCH sitter på insidan av cellens ytmembran och förmedlar signaler från vävnaden utanför cellen och in i cellen och reglerar på så vis cellens förmåga att röra och dela sig. Ett ökat uttryck av PINCH ger en ökad aggressivitetsgrad i tumören och detta har visat sig vara relaterat

till sämre överlevnad hos patienter med tjocktarmscancer. Det här är den första studien som analyserat förhållandet mellan PINCH och strålbehandling på tumörer från patienter. I gruppen som inte fått strålbehandling så kunde vi visa att patienter med ett starkt PINCH uttryck var relaterat till sämre överlevnad jämfört med patienter som hade ett svagt PINCH uttryck. I den strålbehandlade gruppen sågs ingen skillnad i överlevnad mellan de patienter som hade svagt och starkt PINCH uttryck. Hos de patienter som fått strålbehandling sågs ett ökat uttryck av PINCH och en ökad mängd lymfkärl.

Tumörceller sprider sig via blod- och lymfkärl. En ökad mängd blodkärl och lymfkärl har visat sig vara relaterat till sämre överlevnad hos cancerpatienter. Få har tidigare studerat lokaliseringen av blod- och lymfkärl i tumörvävnaden och hur den är relaterad till överlevnad hos cancerpatienter. Det här är den första studien som studerar lokaliseringen av blod- och lymfkärl och deras förhållande till överlevnad hos ändtarmscancer patienter. I hela gruppen av patienter sågs en ökad överlevnad för de patienter som hade lymfkärl i periferin av tumören jämfört med patienter som hade lymfkärl i det inre tumörområdet/invasionsområdet.

Slutligen studerades mängden inflammatoriska celler, ärrvävnad, död vävnad och slem. Vi kunde visa att patienter med mycket inflammatorisk infiltration hade en bättre överlevnad jämfört med patienter med lite inflammatorisk infiltration.

Sammanfattningsvis så kunde vi med utgångspunkt från en klinisk studie som undersökte effekten av strålbehandling visa att survivin, PINCH, lymfkärl och inflammatorisk infiltration var starkt relaterat till överlevnad hos ändtarmscancer patienter. Den här informationen kan förhoppningsvis hjälpa oss i framtiden att förlänga överlevnaden för ändtarmscancer patienter genom att välja ut de patienter som är bäst lämpade för strålbehandling.

Abbreviations

AO	Antisense oligonucleotides
Apaf-1	Apoptotic protease activating factor-1
APC	Adenomatous polyposis coli
ATM	Ataxia telangiectasia mutated
bFGF	Basic fibroblast growth factor
BVD	Blood vessel density
CAFs	Cancer associated fibroblasts
CDK	Cyclin dependent kinases
Cox-2	Cyclooxygenase-2
CRC	Colorectal cancer
DAB	Diaminobenzidine
DC	Dendritic cells
DCC	Deleted in colorectal cancer
Diablo	Direct inhibitor of apoptosis-binding protein with low pI
DMEM	Dulbecco's Modified Eagles Medium
DNA	Deoxyribonucleic acid
DSBs	Double strand breaks
EGF	Epidermal growth factor
EMC	Extracellular matrix
FAP	Familial adenomatous polyposis
Fc	Fragment, crystallizable region
FGF	Fibroblast growth factor
Gy	Gray
HNPCC	Hereditary nonpolyposis colorectal cancer
IAP	Inhibitor of apoptosis protein
IGF-1	Insulin growth factor-1
IGFBP-3	Insulin growth factor binding protein-3
IHC	Immunohistochemistry
IL	Interleukin
ILK	Integrin-linked kinase
INF- γ	Interferon-gamma
LVD	Lymphatic vessel density
LYVE	Lymph-angiogenic markers

M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MSI	Microsatellite instability
MUCs	Mucinous adenocarcinomas
MVD	Micro vessel density
NK	Natural killer cells
NO	Nitric oxides
NSAID	Non steroidal inflammatory drug
PDGF	Platelet derived growth factor
PINCH	Particularly interesting new cysteine-histidine rich protein
PI3	Phosphatidylinositol 3 kinase pathway
Prox-1	Prospero homeobox protein-1
PT	Permeability transitions pore
PVDF	Polyvinylidene fluoride
Rb	Retinoblastoma
Rsu-1	Ras suppressor protein-1
RT	Radiotherapy
SiRNA	Small interfering RNA
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
α -SMA	alfa-smooth muscle actin
SMAC	Second mitochondria-derived activator of caspase
SSBs	Single strand breaks
TAM	Tumor associated macrophages
TGF- β	Tumor growth factor-beta
TME	Total mesorectal excision
TNM	Tumor node metastasis
TUNEL	Terminal deoxynucleotidy transferase-mediated dUTP-biotin nick end-labelling
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

List of papers

This thesis is based on the following papers;

1. **Knutsen (Holmqvist*) A**, Adell G, Sun X-F. Survivin expression is an independent prognostic factor in rectal cancer patients with and without preoperative radiotherapy. *Int J Radiat Oncol Biol Phys* 2004;60:149-155.
2. **Holmqvist A**, Gao J, Holmlund B, Adell G, Carstensen J, Sun X-F. PINCH is an independent prognostic factor in rectal cancer patients without preoperative radiotherapy –A study in a Swedish rectal cancer trial of preoperative radiotherapy. Submitted.
3. **Holmqvist A**, Gao J, Adell G, Carstensen J, Sun X-F. The location of lymphangiogenesis is an independent prognostic factor in rectal cancer patients with or without radiotherapy. *Ann Oncol* 2010;21:512-517.
4. **Knutsen (Holmqvist*) A**, Adell G, Sun X-F. Inflammatory infiltration, fibrosis, necrosis and mucinous content in relation to clinicopathological and molecular factors in rectal cancers with or without preoperative radiotherapy. *Oncol Rep* 2006;16:321-327.

* Married Holmqvist in the year of 2007

Introduction

Colorectal cancer (CRC) is one of the most common malignant diseases in the world. In Sweden there are 5500 new cases each year, where 2000 of these cases are rectal cancers. In the beginning of the twentieth century the local recurrence rate in rectal cancer patients was high and could vary between 20-45% (Phillips et al., 1984). In the 1980s, researchers showed that the combination of improved surgical techniques and short term preoperative radiotherapy remarkably reduced the local recurrence rate to around 5% (Kapiteijn et al., 2001). Even though the local recurrence rate has been reduced, the mortality rate is still high (The National Board of Health and Welfare, 2009) and there are still huge variations in the response to preoperative radiotherapy (RT) in patients with the same tumour stage. Therefore, it is of great importance to identify good predictive and prognostic factors that help us select the best suited patients for preoperative RT in the future.

For many years, studies of neoplastic transformation have focused on the unit of the cell, and their signal transduction pathways, cellular proliferation, death, motility, DNA repair and genomic integrity. In recent years, researchers have begun to realise that the stroma around tumour cells together with their extracellular matrix components also plays an important role in tumour carcinogenesis.

In this thesis, we wanted to investigate the biological factors, survivin and PINCH, and the histological factors, inflammatory infiltration, fibrosis, necrosis, mucinous content, angiogenesis and lymphangiogenesis. Further we wanted to investigate these biological and histological factors in relation to preoperative RT and to clinical variables in rectal cancer patients participating in a clinical trial of preoperative RT.

Background

Epidemiology

CRC is the 3rd most common malignancy in the world with over one million new cases each year. The incidence rate varies 20-30 folds internationally. The highest rates of the disease are seen in industrialised countries such as the USA, Europe, Australia and New Zealand. The lowest incidence is seen in non-industrialised countries as India and Algeria (Boyle & Leon, 2002). In Sweden, there are around 5500 new cases each year where 3500 of these are colon cancer and 2000 rectal cancer (The National Board of Health and Welfare, 2009). It is the second most common cancer in women after breast cancer, and the third most common cancer in men after prostate and lung cancer. During the last decades, the age standardised incidence for rectal cancer patients has increased. The mortality rate is still high, but has decreased slightly since the beginning of 1970, probably due to the combination of preoperative RT, chemotherapy and improved surgical techniques (Kapitejn et al., 2001), (The National Board of Health and Welfare, 2009). Colon cancer mortality is constant since 1995. Data from the National Board of Health and Welfare in 2009 showed that the 5-year survival rates for men with rectal cancer were 57.4% and for women 61.7%. Rectal cancer is rather unusual in young patients and 75% of the patients receives their disease after the age of 65.

Aetiology and risk factors

Many factors such as the environment, lifestyle, previous irradiation, diet, age, inflammatory bowel disease and heredity are associated with CRC (Boyle & Leon, 2002). Ninety to ninety-five percentages of all cancers are caused by environmental factors and life style, and 5-10% is caused by genetic defects (Sutandyo, 2010). In follow-up studies of cohorts and in case-control studies physical activity equivalent to walking 4 hour per week in both men and women was associated with a decreased risk of developing adenomas and CRC (Boyle & Leon, 2002). The reason for such an association has not been identified, but has been postulated as being changes in gastrointestinal transit time, altered immune function and prostaglandin levels as well as changes in insulin levels, insulin-like growth factors, bile acid secretion, serum cholesterol, gastrointestinal and pancreatic hormone profiles (Simon, 1984; Quadrilatero & Hoffman-Goetz, 2003). Both smoking and alcohol consumption have been associated with modestly increased risk of CRC. The risk of developing smoking/alcohol related cancers is dose related, the more cigarettes/alcohol, the higher the risk of cancer. Carcinogens from tobacco could reach the colorectal mucosa through either the gastrointestinal tract or via the circulatory system and damage or alter the expression of cancer-related genes (Boyle & Leon, 2002). Alcohol in the blood is bio-transformed to acetaldehyde in the liver.

Acetaldehyde increases the production of free radicals, which could cause deoxyribonucleic acid (DNA) damage and lead to increased risk of cancer (Kumar et al., 1997).

Another risk factor for developing cancer is RT. It was shown that women who received RT treatment for cervix cancer had an increased risk of developing rectal cancer (Smith, 1962).

Eating habits have been shown to be the main cause of the huge incidence variations between different countries worldwide (Willet, 1995). Much epidemiological evidence shows that compounds in food such as aflatoxin B1, nitrosamines, and polycyclic aromatic hydrocarbons acts as mutagens in the colon and rectum. In addition, excessive intake of fatty acids, red meat and calories also raises the cancer risk, while intake of fruit, vegetables and fish reduces the cancer risk (Willett, 1995; Sutandyo, 2010). A high fat intake increases the production of cholesterol and bile acids from the liver, which could be converted to mutagens by bacteria in the bowel (Bernstein et al., 2009). It has been postulated that dietary fibres protects against CRC by absorbing or diluting bile acids and neural sterol metabolites produced by interstitial bacteria (Reddy et al., 1989). Dietary fibres also increase the volume of the stool and protects against faecal retention of the bowel (Huang, 1978; Freeman, 1979).

Calcium, vitamins, hormone substitution in women after menopause and non-steroidal anti-inflammatory drugs acts as protectors against CRC (Willet, 1995; Boyle & Leon, 2002).

Patients with chronic inflammation of the large intestine have a 20% increased risk of developing cancer. In patients with ulcerous colitis the risk increases after 10 years and the incidence at that time is 5-6 times higher compared to patients without inflammation in the bowel. In patients with Morbus Chron before the age of 30, and in patients with diverticulitis there is also an increased risk of cancer.

Insulin-like growth factor-1 (IGF-1) has been reported to correlate with increased risk of cancer in several sites (colon, prostate, breast and lung), and high levels of circulating IGF-1 and low levels of insulin growth factor binding protein-3 (IGFBP-3) were associated with an elevated risk of tubulo-villous/villous colorectal adenoma and cancer (Franceschi et al., 2001).

Heredity

Ten to twenty percent of all CRC cases are associated to a family history. Most of them are autosomal dominant, which means that the risk for a first-degree relative to receive the disease is 50%. In some of these inherited cancers the genetic changes are known.

Hereditary non-polyposis colorectal cancer (HNPCC) has the clinical features of autosomal dominant heredity, usually with an early (mean age 40 years) and fast development (within 1 year) of CRC. It accounts for at least 2% of all CRC and is often located on the right colon as multiple polyps. Carriers have a lifetime risk of 80% of developing colorectal and 60% risk of developing endometrial cancer (for women). There is also an increased risk to develop other cancers such as; ovarian-, biliary tract-, urothelial-, central nervous system (CNS) and ventricle

cancer (Aarnio et al., 1999; Loukola et al., 1999). In HNPCC patients, mutations occur in the DNA mismatch-repair genes and give rise to microsatellite instability (MSI). As a result of the mutations, MSI can be detected. The patients with HNPCC have a better prognosis compared to patients with sporadic CRC.

Familial adenomatous polyposis (FAP) is an autosomal dominant syndrome that accounts for less than 1% of all CRC. The main clinical features are the development of hundreds to thousands of small adenomas of the colon and rectum usually before the age of 30 years. If the polyps are not removed, cancer will develop before the age of 40 years. Classic FAP is inherited in an autosomal dominant manner and results from a germ-line mutation in the adenomatous polyposis coli (APC) gene. FAP may present with some extra-intestinal manifestations such as osteoma, dental abnormalities, desmoids tumors and extra-colonic cancers (thyroid, liver, bile ducts and CNS). By the late teens or early twenties CRC prophylactic surgery is usually performed (Half et al., 2009).

Other disorders that could cause multiple polyps in the colorectum include; Peutz-Jeghers syndrome, familial juvenile polyps, hyperplastic polyposis, hereditary mixed polyposis syndromes and Lynch syndrome (Half et al., 2009).

Pathology

The development of carcinoma from adenomatous lesions is referred to as the adenoma-carcinoma sequence. It starts in a normal epithelial cell of the colorectal mucosa. Loss of one normal copy of the cancer suppressor gene APC and loss of DNA repair genes (either inherited or acquired) occur early and is called “first hit” according to Knudson et al (1971). An adenoma starts to develop when the loss of the second normal copy of APC or DNA repair genes follows (“second hit”) together with hypo-methylation of the DNA. Adenomas are true neoplastic lesions and are precursors of carcinoma. Further mutations of the oncogene K-ras occurs and additional mutations inactivate the tumor suppressor genes deleted in colon carcinoma (DCC) and p53 which finally leads to the emergence of carcinoma (Kumar et al., 1997), (Figure 1).

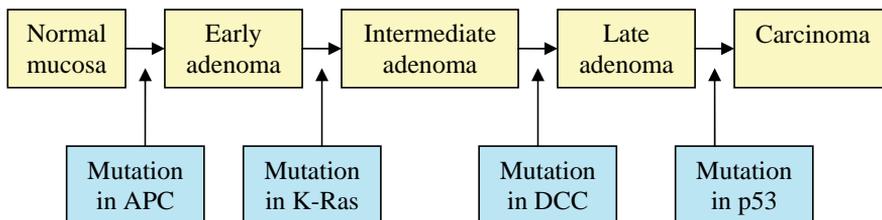


Figure 1. The adenoma-carcinoma sequence (Fearon & Vogelstein, 1990).

Ninety-five to ninety-eight percent of the CRC malignancies are adenocarcinomas and 10-20% of the adenocarcinomas that comprise more than 60% mucus are referred to as mucinous adenocarcinomas (MUCs). Less frequent histological types include signet-ring cell carcinoma (1%), squamous carcinoma (usually originating from the stratified squamous epithelium of the anal canal), undifferentiated carcinoma and medullary-type carcinoma (Kumar et al., 1997; Ponz de Leon & Di Gregori, 2001). Carcinoid tumours compromise less than 2% of colorectal malignancies, but almost half of tumours in the small intestine (Kumar et al., 1997).

The histological grading for CRC is well differentiated, moderately differentiated and poorly differentiated lesions (Bosman, 1995). Well-differentiated cancers resemble very closely their normal counterparts, with few mitoses; growths in a well defined glandular pattern and have regularly shaped tumour cells. Moderately differentiated cancers have more irregularly shaped glandular pattern and less regularly shaped cells than well-differentiated cells. Poorly differentiated tumours have a marked nuclear and cellular pleomorphism with numerous, distinctly atypical mitoses, loss of normal polarity and irregular growth pattern.

There are two distinct pathological growth patterns that describe how the tumour cells invade the normal tissue. CRC grows within the tissue either by expanding or infiltrating the normal mucosa. The expansive type has a sharply defined and circumscribed growing margin and is related to better survival compared to the infiltrative type that has no recognisable margin of the growth (Washington, 2008). The infiltrative mode of growth makes it necessary to remove a wide margin of surrounding normal tissue when surgical excision of a CRC is attempted.

Adenomas can be pedunculated tubular-, villous or tubulo-villous. The malignant risk for an adenoma depends on polyp size, histological architecture and severity of the epithelial dysplasia. Cancer is rare in adenomas smaller than 1 cm. The most important risk factor is the number of adenomas and the highest malignant potential has the villous adenoma, where invasive cancer is found in up to 40% of the patients (Kumar et al., 1997) and.

CRC could appear in the cecum, colon ascendens, transversum, descendens, sigmoideum and rectum. About 70% of these lesions are located in the left colon (Ponz de Leon & Di Gregori, 2001). During the past decades the distribution of cancers of the colo-rectum appears to be changing with a shift towards the right colon (Ponz de Leon et al., 2007). It is still unclear if this is a true biological event or if it is the consequence of a wider use of colonoscopy. The transition from the rectum to the colon is defined as 15 cm from the anal verge at the level of the third sacral vertebral. The blood supply to the rectum is derived from branches of the superior mesenteric artery. The large majority of venous blood leaves the colo-rectum through the portal system and reaches the liver, which is the main site of haematogenous metastasis (Ponz de Leon & Di Gregori, 2001). Both the venous and arterial system are closely connected to the lymphatic system where the most common sites for lymphatic drainage is to the

para-aortal lymph nodes, lymph nodes at the side walls of pelvis, lymph vessels around anus or inguinal nodes.

Staging of CRC is based on the tumor stage (T-stage), lymph node stage (N-stage) and the presence of distant metastases (M-stage). In the beginning of the 1930 century, Dukes' developed a widely used morphological classification system for CRC. During the recent decades the TNM system has become increasingly popular and is nowadays universally accepted as the classification system for CRC (Table 1), (Sobin et al., 2009). It has been shown that differentiation grade is associated with patients survival (Deans et al., 1994) and nowadays the TNM stage together with the differentiation grade serves as a guide for deciding the treatment for CRC patients (Zlobec & Lugli, 2008; Gravalos et al., 2009).

Table 1. TNM classification and 5-year survival in CRC patients

TNM staging	Description	Dukes' stage	5-years survival
I	T1, N0, M0	T1 Tumour invades submucosa	A 80-95%
	T2, N0, M0	T2 Tumour invades muscularis propria	
II A	T3, N0, M0	T3 Tumour invades through muscularis propria into subseros or into non peritonealised pericolic /rectal tissue	B 60-80%
II B	T4, N0, M0	T4 Tumour directly invades other organs, structures or perforates the visceral peritoneum	
III A	T1, T2, N1, M0	N1 Metastasis in 1 to 3 regional lymph-nodes	C 30-55%
III B	T3, T4, N1, M0		
III C	Any T, N2, M0	N2 Metastasis in 4 or more regional lymph- nodes	
IV	Any T, Any N, M1	M1 Distant metastasis	D <5%

A definite staging of the tumour can only be made postoperatively, a good preoperative investigation for CRC patients is also important for selecting therapies. The preoperative local staging for rectal cancer is based on endorectal ultrasound and magnetic resonance imagine (MR) of the pelvis, the presence of distant metastasis is assessed by ultrasound of the liver, chest x-ray or computerised tomography (CT) of the abdomen (Roberts, 1999).

Histological factors

Inflammatory infiltration

Inflammatory infiltration is a complex system that plays a paradoxical role in the development of CRC. It has been shown that patients with chronic inflammatory bowel disease have an increased risk of developing CRC (Coussens & Werb, 2002; Kulaylat & Dayton, 2010) while in patients with CRC, a positive relationship between inflammatory infiltration and good prognosis has been found (Nagtegaal et al., 2001; Shia et al., 2004; Gao et al., 2005). In tumours, this could be caused by acutely activated immune cells that contribute to T-lymphocyte (T-Cell) activation, while in patients with chronic inflammatory bowel disease; chronically activated immune cells causes T-cell dysfunction through the production of reactive oxygen (de Visser et al., 2006). In head and neck cancer, an aggravation of inflammatory infiltration induced by interleukin-15 (IL-15) was related to poor prognosis, while infiltration of regulatory T-cells was beneficial for local control of the tumour (Badoual et al., 2006; Fridman et al., 2010). In other cancers, such as breast and pancreas cancer, inflammatory infiltration was associated with a worse prognosis (Emmrich et al., 1998; Murri et al., 2008).

Tumour cells can escape the immune defence by decreasing their expression of co-stimulating factors, decreasing major histocompatibility complex (MHC) class I molecules, reducing the production of antigen or adhesion proteins, or by producing growth factors (Kumar et al., 1997).

Other factors involved in the immunological reaction around tumour cells are cytokines, growth factors, interferons, matrix metalloproteases (MMP), cyclooxygenase-2 (Cox-2) and nitric oxides (NO) (Brigati et al., 2002).

Tumours are commonly infiltrated by T-lymphocytes, B-lymphocytes (B-cells), cytotoxic-T cells, Natural Killer (NK) cells, macrophages, dendritic cells (DC), neutrophils, eosinophils, basophils and mast cells.

The T-cells play a key role in tumour immunity. They develop in the bone marrow and migrate to the thymus where they mature into either a CD4+ or CD8+ cell. The CD8+ cell plays an important role in tumour cell surveillance. It is activated by the recognition of a foreign antigen on the MHC class I receptor, which leads to lysis of the cell, directly via the fas/perforin pathway or indirectly via the release of cytokines. CD8+ cells have been shown to be associated with longer survival and a higher apoptotic index in CRC (Dolcetti et al., 1999). CD 4+ cells are called helper cells and are involved in the activation of B-lymphocytes. In animal studies of cervix cancer it was shown that an elimination of CD 4+ lymphocytes increased the tumour burden (Daniel et al., 2005).

B-cells proliferate in the draining lymph nodes, migrate into the tumour and are activated by CD4+ cells, resulting in the production of antibodies. These antibodies can trigger activation of immune cells by the cross linking of fragment crystallizable (Fc) receptors or by the activation of complement (Hoebe et al., 2004). Early studies have shown that passive transfer of tumour specific antibodies increases outgrowth of transplanted tumour cells (Agassy-Cahalon et al., 1988), whereas the absence of B-lymphocytes limits tumour formation (Brodt & Gordon, 1982).

The NK cells are lymphocytes that are capable of destroying tumour cells, virus infected cells and some normal cells without prior sensitisation. They may provide the first line of defence against tumours. NK cells are activated by interleukin-2 (IL-2), and use two major mechanisms to induce target cell apoptosis, either by the granule exocytose pathway (by a membrane disruption protein called perforin) and the death receptor pathway (Wallace & Smyth, 2005). NK cells are inactivated by normal cells that express MHC class I molecules. T-cells and NK cells seems to provide complementary anti-tumour mechanisms. Tumours that fail to express MHC class I antigens can not be recognised by T-cells, but these tumour cells may trigger NK cells (Kumar et al., 1997). In a recent cell line study, NK cells were shown to prefer tumour cell killing before killing normal cells (Smyth et al., 2001) and in CRC, strong infiltration of NK cells was related to better survival (Coca et al., 1997).

Macrophages are a major component of innate (non-specific) immune cells. They are derived from blood monocytes and can either differentiate into different resident tissue macrophages or travel around in the lymph system. They are important in mediating the tissue destruction, angiogenesis and fibrosis characteristic of chronic inflammation (Kumar et al., 1997) and are activated by interferon- γ (INF- γ) secreted by T-lymphocytes. Macrophages have two functions; the first is to catch foreign material, break them down, travel to the closest lymphoid organs and present the antigen for T-cells, the second is to identify antibodies and complement located on foreign material and engulf them (Brändén, 1995). Recent studies have shown that the tumour associated macrophages (TAM) have dual roles in tumour development, they have been reported to kill tumor cells, but they can also activate the coagulatory system, be immunosuppressive, stimulate tumour growth, and produce angiogenetic factors and MMP (Brigati et al., 2002). Previously, it was shown that CD-163, a special marker for macrophages, was highly expressed in cells of rectal and breast cancers, suggesting that metastasis of tumour cells occurs after fusion with macrophages (Shabo et al., 2009).

DCs (Dendritic cells) are a part of a population of cells that have dendritic cytoplasmic processes and large amount of class II molecules on their cell surface. They are found in lymphoid tissues and migrate to peripheral sites where they activate T-cells (Brigati et al., 2002; Kumar et al., 1997). In tumours, the maturation of DC was shown to be inhibited by IL-6 and macrophage colony-stimulating factor (M-CSF), making it difficult for DC to activate T-cells

(Katsenelson 2001). Therefore, specific therapies for DC stimulation, such as IL-4, try to prevent tumours avoiding immunosurveillance (Menetrier-Caux et al., 2001).

The relationship between inflammatory infiltration and RT is still not yet clear. It was shown that low-dose RT used as a treatment for painful joint diseases had a pronounced anti-inflammatory effect; while in contrast, the high dose of RT given in cancer therapy induced the expression of pro-inflammatory cytokines (Hallahan et al., 1996, Hildebrandt et al., 1998).

Fibrosis

Fibrosis is most commonly initiated by a severe and persistent tissue injury with damage of normal tissue and recruitment of inflammatory cells. The inflammatory cells produce growth factors, such as platelet-derived growth factor (PDGF), tumor growth factor- β (TGF- β) and basic fibroblast growth factor (b-FGF), which promote fibroblast migration and proliferation. The fibroblasts synthesise extra cellular matrix (ECM) mainly composed of collagen, which is deposited between the cells as fibrosis (Kumar et al., 1997).

It is suggested that most of the fibroblasts in the tumor tissue are “activated” fibroblasts with typical signs of smooth muscle differentiation called cancer associated fibroblasts (CAFs) (Gabbiani et al., 1971). CAFs are morphologically characterised by large spindle-shaped cells with indented nuclei. In cancers, CAFs are normally defined by the concurrent expression of α -smooth muscle actin (α -SMA) and vimentin (Arora & McCulloch, 1999). CAFs usually originate from already existing fibroblasts, but could also originate from the vascular bed (Rønnev-Jenssen et al., 1995) or from epithelial tumor cells (Petersen et al., 2003). Exactly, what role the CAFs play in tumour development is still not known. Studies of prostate cancer patients and CRC patients showed that CAFs were associated with increased tumour growth and worse survival (Olumi et al., 1999; Tsujino et al., 2007), while others found an association between CAFs and reduced tumour growth (Parrott et al., 2001). In CRC patient fibrosis was proposed to have a growth limiting effect on tumour cells and it was also shown that fibrosis was related to improved survival (Adachi et al., 1989; Ueno et al., 2003). RT was suggested to increase the production of TGF- β which in turn promotes the proliferation of fibroblasts (Martin et al., 2000), further the fibroblasts were shown to increase their production of collagen type I and III induced by RT (Rieeki et al., 2002).

Necrosis

Coagulative necrosis is caused by chronic ischemia of the cell and is an exogenous irreversible injury that leads to cell swelling, denaturation of cytoplasmatic proteins and enzymatic digestion. In tumours, coagulative necrosis could be initiated by a rapid tumour growth without sufficient blood supply, which further leads to ischemia and necrosis, or by increased secretion of tumor necrosis factor (TNF) that decreases the blood perfusion and induced hypoxia by activating the coagulation cascade (Raza et al., 2002), or induced by RT (Aki et al., 2001). Increased hypoxia and necrosis have been shown to correlate with resistance to RT and

chemotherapy, increased metastatic potential and worse prognosis in tumours (Vanselow et al., 2000; Swinson et al., 2002; Gao et al., 2005). An important feature of necrosis is that unlike apoptosis, necrosis initiates a pro-inflammatory response with recruitment of cytotoxic immune cells that damage normal tissue and produce mitogenic or pro-survival cytokines (Ricci & Zong, 2006). These cytokines activate signalling pathways that promote cell out-growth and induce cell migration which is further associated with tumour cell survival and metastasis (Lotze & Tracey, 2005).

Mucinous content

The term mucinous means that the tumour tissue has a lot of mucous. In CRC, adenocarcinomas that are comprised of at least 60% of mucus are referred to as mucinous adenocarcinomas (MUCs). MUCs account for about 10-15 % of all adenocarcinomas. They are characterised by a low apoptotic and low proliferative activity (Zhang et al., 1999), and are related to worse outcome in CRC (Akino et al., 2002). MUCs have been shown to be resistant to chemotherapy by overexpressing markers of resistance to both 5-fluorouracil (5-Fu) and oxaliplatin (Eloxatin) (Glasgow et al., 2005). Previous studies have found an increased amount of mucin pools in surgical specimens of patients treated with long course preoperative radio-chemotherapy (Shia et al., 2004) and short course RT (Nagtegaal et al., 2002), suggesting that radio-chemotherapy could increase the production of mucin in tumours.

Angiogenesis

Angiogenesis is the formation of new blood vessels from the existing vascular bed. It is normally suppressed and is observed only transiently during reproduction, tissue development and wound healing. Sustained angiogenesis is characteristic of diseases such as diabetes, psoriasis and rheumatoid arthritis. Although it has been recognised that most solid tumours contain a large number of blood vessels, the importance of these vessels in tumours was first discovered by Folkman in the early 1970s. He hypothesised that the new vessels were required for expansion when the tumour reached a size of 1-2 mm. At that point diffusion of nutrients and waste products became limiting for further tumour growth (Folkman, 1971). The first molecule definitively identified as a pure angiogenic factor was basic fibroblast growth factor (b-FGF), which was followed by the identification of a large number of angiogenic factors produced by tumour cells themselves or by immune cells in the tumour tissue (O'Byrne et al., 2000). Recent attention has focused on members of the fibroblast growth factor (FGF) and the vascular endothelial growth factor (VEGF) families which have shown to be the most important factors in tumour angiogenesis (Fernig & Gallagher, 1994; Dvorak et al., 1995). The VEGF family consists of VEGF-A to VEGF-D and binds variably to three high-affinity endothelial cell tyrosine kinase receptors called vascular endothelial growth factor receptor 1-3 (VEGFR1-3). This growth factor receptor complex increases vascular permeability, endothelial cell proliferation and tube formation. The most important growth factor

has been shown to be VEGF-A, which is highly expressed in many types of tumours and is strongly associated with microvessel density (MVD) and survival (Fox et al., 2001). When tumours do not use VEGF-A other VEGF homologues may be induced (Joukov et al., 1997). MVD has been shown to be a powerful prognostic indicator in many types of tumours (Folkman, 2002; Des Guetz et al., 2006). There are several angiogenesis related markers that measure intra-tumoural MVD (Fox et al., 2001). In CRC, most studies have used antibodies against factor VIII antigens, which stain mainly mature vessels and cross-react with lymphatic endothelium. In several recent studies, antibodies directed against CD34 and CD31 showed a strong association between MVD and survival, suggesting that these antibodies are good prognostic markers in CRC tumours (Des Guetz et al., 2006).

The relationship between angiogenesis and RT is still a subject for discussion. In studies of rectal cancer and retinal endothelial cells, RT was suggested to destroy vascular integrity and reduce MVD (Baeten et al., 2006; Mao, 2006). It was shown that the expression of VEGF was increased after RT, and that increased endothelial cell killing occurred when VEGF antibodies were combined with RT (Gorski et al., 1999). Others showed that RT increased the levels of NO and increased the angiogenesis in tumours (Sonveaux et al., 2003).

Lymphangiogenesis

The lymphatic vasculature forms a vessel network that drains interstitial fluid from tissue and returns it into the blood. Lymphatic vessels are also an essential part of the body's immune defence. They play an important role in the pathogenesis of several diseases, such as cancer, lymph-oedema and various inflammatory conditions. The lymphatic vessels are thin-walled, relatively large vessels, composed of a single layer of endothelial cells. Lymphatic capillaries compared to collecting lymphatic vessels are not en-sheathed by pericytes or smooth muscle cells and do not have valves and little or no basement membrane. The contraction of surrounding skeletal muscles contributes to lymph fluid propulsion, where the valves prevent backflow of the fluid (Alitalo et al., 2005). The lymphatic endothelial growth is mainly stimulated by VEGF-C and -D (Yonemura et al., 2005; Miyata et al., 2006) by binding to their receptor VEGFR-3 (Yonemura et al., 2005). In a cell line study of human lung cancer, it was shown that VEGF-C by binding to its receptor (VEGFR-3) stimulated the lymphatic sprouting towards tumour cells as well as the dilatation of the pre-existing lymph-vessels, suggesting that VEGF-C and its receptor plays an active role in the lymphatic spread (He et al., 2005). In recent years the relationship between lymphatic vessel density (LVD) and prognosis has been studied extensively, however, the findings are not consistent. In studies of CRC, breast, oesophagus and gastric cancer a high level of LVD was positively related to lymph-node metastasis and worse prognosis (Bono et al., 2004; Nakamura et al., 2006; Saad et al., 2006; Inoue et al., 2008) while others found the reverse relationship (Maula 2003). The relatively new lymph-angiogenetic markers lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1),

podoplanin, prospero homeobox protein 1 (Prox-1), 5'-nucleotidase and D2-40 are able to distinguish lymphatic vessels from blood vessels. Their different abilities could explain the diverse results between LVD and prognosis. LYVE-1 has been questioned in identifying fully functional lymphatic vessels, while Prox-1 mostly identifies lymph vessels in normal tissue and 5-nucleotidase enzyme activity is observed in both blood and lymph vessels (Parr & Jiang, 2003). D2-40, compared to the other markers mentioned, identifies lymph-vessels in lamina propria of colon tumours, suggesting that D2-40 is a better marker to identify immature lymphatic vessels (Foght et al., 2003). Few have studied the relationship between lymph-angiogenesis and RT in normal tissue or in tumours. One recent study showed that LVD was increased in normal skin samples taken one year after radiation (Jackowski et al., 2007).

Biological factors

Survivin

Survivin is normally involved in the healing process of tissue injury where it inhibits apoptosis and regulates the cell cycle. It is preferentially expressed at mitosis dealing with the formation of microtubuli (Rosa et al., 2006). Survivin belongs to the inhibitor of apoptosis family (IAP) and consists of a protein of 142 amino acids with a molecular weight of 16.3 kDa. It blocks apoptosis by inhibiting activation of pro-caspase 9 (Altieri, 2006), caspase-3 and -7 (Shin et al., 2001), (Figure 2) and regulates the cell cycle in the G2/M phase. The function of survivin is regulated by the pro-apoptotic protein second mitochondria-derived activator of caspase/direct inhibitors of apoptosis-binding protein with low pI (Smac/DIABLO). Induction of apoptosis leads to the release of smac/DIABLO which prevents survivin from inhibiting caspase-3 and -7 (Figure 2), (Anguiano-Hernandez et al., 2007). Survivin is expressed during embryonic development as well as in the majority of human cancers, but is undetectable or weakly expressed in normal adult tissues (Ambrosini et al., 1997; Adida et al., 1998). Survivin is related to worse prognosis in several types of tumours (Kawasaki et al., 1998; Lei et al., 2010; Yang et al., 2010). Recent studies have shown that survivin interacts with the transition from low dysplasia to high dysplasia in colorectal adenomas (Gianani et al., 2001) and is related to APC (Zhang et al., 2001), suggesting that survivin is an early event in tumour development. Survivin was also associated with nuclear factor- κ B (NF- κ B), which leads to increased tumour cell invasion and metastasis (Mehrotra et al., 2010). Survivin has critical functions in preserving endothelial cell viability during the proliferative phase of angiogenesis (Sakao et al., 2005). Another protein of interest connected to survivin is p53. It was shown that wild-type p53 suppressed survivin expression both at messenger ribonucleic acid (mRNA) and protein levels (Hoffman et al., 2002; Mirza et al., 2002) and that DNA damage

induced a p53-survivin signalling pathway that regulated the cell cycle and apoptosis (Zhou et al., 2002).

Because of its up-regulation in malignancy and its key role in apoptosis, proliferation and angiogenesis, survivin nowadays attracts attention as a new target for anti-cancer therapies. In several animal models, inactivation of survivin with antisense oligonucleotides (AO) has been shown to inhibit tumour growth (Kanwar et al., 2001; Tu et al., 2003). Furthermore, AO-mediated down-regulation of survivin in cancer cells enhanced sensitivity to cisplatin (Kojima et al., 2006), taxol (Fisker et al., 2007) and etoposid (Sharma et al., 2005). AO-mediated down-regulation of survivin has also been reported to increase sensitivity to RT (Sah et al., 2006). Right now there are several ongoing phase I and II clinical trials that further investigate the role AO in cancer treatment. Other strategies under investigation to target survivin include small interfering RNA (siRNA), ribozymes and immunotherapy (Ryan et al., 2009).

The relationship between survivin and RT in tumours has been studied by others, where survivin was shown to be up-regulated and resistant to RT (Asanuma et al., 2000; Rödel et al., 2002; Rödel et al., 2003; Lu et al., 2004; Rödel et al., 2005).

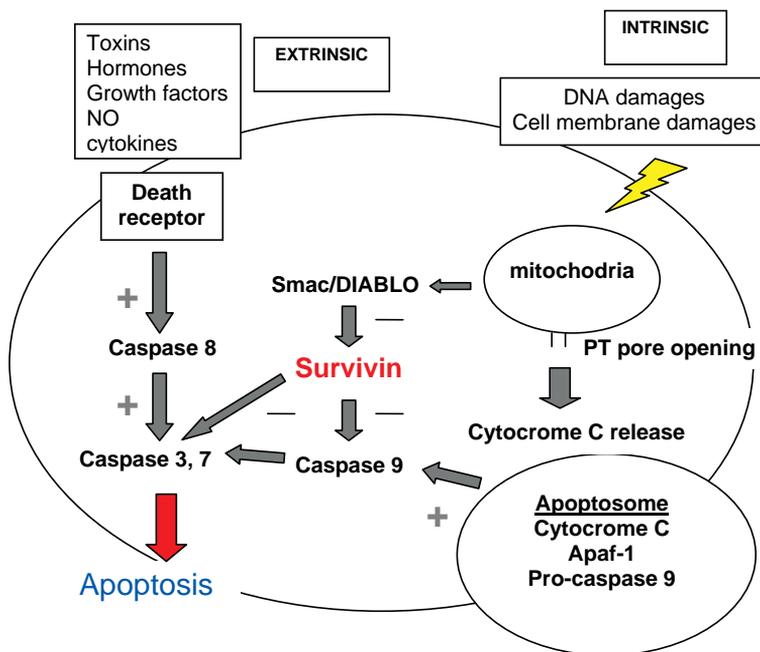


Figure 2. Survivin in relation to apoptosis

PINCH

Particularly interesting new cysteine-histidine rich protein (PINCH) is a five LIM domain protein of 35.8 kDa with a gene located on chromosome 2q12.2. It is a part of the PINCH-ILK-Parvin complex connected to integrins of the cell surface, and acts as an adapter protein for signal transduction through the cytosol (Rearden, 1994). PINCH creates together with integrin-linked kinase (ILK) and parvin a (PIP) complex, which provides crucial physical linkages between the actin cytoskeleton and transduces signals from the extracellular matrix to intracellular effectors (Tu et al., 1998; Tu et al., 1999). These effector proteins further regulate the cytoskeleton organisation, spreading, motility and proliferation of the cell (Webb et al., 2002; Fukuda et al., 2003). PINCH is suggested to regulate cell proliferation by inhibiting apoptosis. It was shown that PINCH increased the phosphorylation of PKB/Akt which further decreased the activity of caspase-3 (Fukuda et al., 2003), while others showed that PINCH contributed to apoptosis resistance by suppressing the ERK-Bim pathway in sarcoma cell lines (Chen et al., 2008). Recently, it was shown that PINCH had other important functions. Tu et al (1998) showed that NCK-2 interacted with both PINCH and the epidermal growth factor receptors (EGF) and PDGF, suggesting that NCK-2 serves as an adaptor protein connecting the growth factor receptor pathways with the integrin-receptor pathway. Others showed that Ras suppressor protein-1 (Rsu-1) linked the Ras pathway with the PIP complex (Dougherty et al., 2008). Most of the PINCH expression in tumours and in normal tissue is located in the cytoplasm of fibroblasts, but also to a small extent in epithelial cells of tumours and normal tissue. PINCH was shown to increase from normal mucosa to primary tumour to metastasis. In primary CRC tumours, the most intense PINCH staining was found at the invasive edges (Wang-Rodriguez et al., 2002). A strong PINCH expression was also related to worse prognosis in CRC patients (Gao et al., 2006). Recently, two structurally closely related proteins have been identified called PINCH-1 and PINCH-2. They are encoded by two different genes and shares 82% significant sequence similarity at the amino acid level (Zhang et al., 2002). Both PINCH-1 and PINCH-2 are widely expressed in normal mammalian cells. PINCH-1 unlike PINCH-2 is expressed in embryonic cells, in the spleen and thymus. There are at least two distinctive PIP complexes, one containing PINCH-1 and the other containing PINCH-2 (Zhang et al., 2002). A depletion of PINCH-1 from human cells devastated cell spreading and survival, further, a high expression of PINCH-2 was unable to rescue the phenotypical defects induced by the loss of PINCH-1 (Fukuda et al., 2003). Very few have investigated the relationship between PINCH and RT. Recently, one cell line study of mouse fibroblasts and human colon, lung, cervix, skin and pancreas tumours showed that PINCH enhanced radio-resistance by inhibiting PP1 α via the Akt-1 pathway (Eke et al., 2010).

Apoptosis

Apoptosis, or “programmed cell death” is a physiological cell suicide mechanism that plays a central role in both development and homeostasis in adult tissue. The morphological features of the apoptotic cells are cytoplasmic shrinkage, membrane blebbing, and condensation of chromosomes, DNA fragmentation and the formation of apoptotic bodies. Further these bodies are phagocytised by macrophages, preventing an inflammatory response (Peltenburg, 2000). The activation of the biochemical killing can be induced by various signals. Withdrawal of growth factors or hormones and receptor-ligand interactions is initiated at the cell surface and is propagated via the cytosol to the mitochondria, DNA damage or intrinsic protease activation (during embryogenesis) occurs through nuclear and mitochondrial routes (Kumar et al., 1997). This further leads to the efflux of cytochrome C from the mitochondrion which is a crucial step in the apoptotic process. Cytochrome C together with the apoptotic protease activating factor-1 (Apaf-1) and procaspase-9 form an apoptosome which then activates caspases that further degrade protein components of the cell (Figure 2), (Green & Reed, 1998). The main effector caspases are 3, 6 and 7 (de Bruin & Medema, 2008). The cytochrome C release is caused by a collapse of the inner transmembrane potential of the mitochondria, which leads to the opening of a large conductance channel known as the permeability transition (PT) pore (Green & Reed, 1998). Anti-apoptotic proteins such as Bcl-2 and Bcl-X_L blocks the cytochrome C release by modulating the PT pore, while pro-apoptotic proteins such as C-MYC allows cytochrome C to pass through the mitochondrial membrane (Juin et al., 1999). Apoptosis initiated by p53 involves elevated level of a pro-apoptotic gene called Bax. Other ways to regulate apoptosis are via the Raf-MAP kinase route or the phosphatidylinositol 3 (PI3) kinase pathway, the MAP kinase pathway is associated with either positive or negative effects of apoptosis while the PI3 kinase pathway has been strongly connected with protection from apoptosis via PKB/Akt (Downward, 1998). The family of IAP block apoptosis mostly by inhibiting caspase 3 and 7 (Roy et al., 1997) and a member of this family is survivin (Ambrosini et al., 1997). IAP is further regulated by Smac/DIABLO which is released by mitochondria in response to apoptotic stimuli and is thought to regulate apoptosis by antagonising IAP (Wu et al., 2000). RT induced apoptosis could be caused by disruption of the electron transport in mitochondria which leads to oxidative stress and PT pore opening (Garcia-Ruiz et al., 1997), via mitotic cell arrest caused by DNA damage, or by increased intracellular Ca⁺⁺ caused by cell membrane injury which leads to direct activation of caspases in the cytosol (Kumar et al.,1997). Failure to undergo apoptosis can result in resistance to both chemotherapy and RT (Meyn et al., 1996; Bergman & Harris, 1997).

p53

p53 is the most common target for genetic alteration in human cancers. The gene is located on chromosome 17p13.1 and consists of 11 exons of which exon 2-11 are transcribed into a protein of 393 amino acids with a molecular weight of 53 kDa (Levine, 1993). p53 is involved in

DNA repair, cell cycle arrest and programmed cell death, genomic stability and blood vessel formation (Chang et al., 1995; Vogelstein et al., 2000). Homozygous loss of the p53 gene is found in virtually every type of cancer. In most instances, mutations that inactivate both copies of the p53 gene are acquired in somatic cells. Less commonly, some individuals inherit a mutant p53 allele which predisposes individuals to develop cancer called the Li-Fraumeni syndrome.

Normal p53 acts in the nucleus and has the ability to inhibit the cell cycle. It applies emergency brakes when the DNA is damaged by mutagenic chemicals, oncogenes or ionising radiation. The normal p53 protein rapidly accumulates in the nucleus and causes cells to arrest in G₁ phase. At the same time p53 induces the transcription of an inhibitor of cyclin dependent kinases (CDK) called p21. This protein prevents the phosphorylation of retinoblastoma protein (Rb) necessary for cells to enter the S-phase. p53 induces a pause in the cell cycle and activates transcription of DNA repair enzymes. If the damage in DNA is repaired, the cell is allowed to complete the cycle, if the repair mechanism fails, normal p53 stops the cell from dividing and induces apoptosis (Levine et al., 1994).

Wild-type p53 could either be p53 with normal function or p53 with mutations altering the reading frame. The half time of wild-type p53 is short and the protein can not be detected by normal immunohistochemistry (IHC) (Cunningham et al., 1992). However, if the protein is inactivated either by missense mutations (a change of one amino acid for another), sub-cellular localisation or binding by viral proteins, it accumulates and can be detected by IHC (Purdie et al., 1991; Levine et al., 1994). It was shown that patients with wild type p53 were more sensitive to RT (Fei et al., 2002). A recent study by ours on rectal cancer patients showed that patients with negative (wild type) p53 had less local recurrence after RT compared to patients with positive p53 (Adell et al., 1999). Mutations of the p53 gene occur in 70% of CRC and it appears to be a late event in the development of sporadic CRC (Fearon & Vogelstein, 1990; Levine et al., 1994).

Cox-2

Cyclooxygenas (Cox) is an enzyme activated by the release of phospholipids from the cell membrane caused by mechanical, chemical and physical stimuli, or by inflammatory mediators (Kumar et al., 1997). The membrane phospholipids consist of aracidon acid which is converted by Cox to prostaglandins which is mainly involved in the inflammatory response. There are two isoforms of Cox; Cox-1 and Cox-2. Cox-1 is produced in most normal tissues, especially the mucosa of the ventricle where it is believed to protect against gastric damage (Sinicrope & Gills, 2004). Cox-2 was first identified in the late 1980s in src-transformed cells of chicken embryos (Xie et al., 1991). It is located in the cytoplasm of the cell and consists of 603 amino acids with a molecular weight of 71kDa. In previous studies, Cox-2 has been shown to be over-expressed and related to worse survival in human CRC as well as in several

other epithelial malignancies (Eberhart et al., 1994; Soslow et al., 2000; O'Connor et al., 2004). Studies on gastric cancers have shown that Cox-2 was involved in the regulation of apoptosis, angiogenesis and tumor cell invasiveness (Fu et al., 2004; Mao et al., 2007).

Multiple studies have shown that non-steroidal anti-inflammatory drugs (NSAID) can prevent experimental colon cancer development. In a study by Kawamori et al (1998) dietary administration of celecoxib in a rat model both reduced the incidence and multiplicity of colon tumours, and it was suggested that the chemo-preventive effect was achieved during the later stages of colon tumour development (Reddy et al., 2000).

Cox-2 was suggested to be up-regulated and enzymatically activated by RT, resulting in elevated levels of prostaglandin E₂ (Steinauer et al., 2000). It was shown that an inhibition of Cox-2 improved tumour response to RT without affecting the surrounding normal tissue (Milas, 2003). In a recent study by ours on the same series of cases (as in this thesis), patients with negative Cox-2 were related to less local recurrence after RT compared to patients with positive Cox-2 (Pachkoria et al., 2005).

Cell cycle

Cellular proliferation is largely regulated by biochemical factors produced in the local micro-environment that can either stimulate or inhibit cell growth. The most important regulatory control is the induction of resting cells in G₀ to enter the cell cycle.

The cell growth cycle consists of the G₁ phase (presynthetic), S-phase (DNA synthesis), G₂ (pre-mitotic) and the M-phase (mitotic). After cell division, the cell can either directly re-enter the cell cycle or proceed to G₀ (Kumar et al., 1997) (Figure 4).

Ionising radiation delays the normal progression through the cell cycle and causes cell cycle arrest in the G₁, G₂ and S-phase. Arrest of the cell cycle at the G₁ checkpoint is caused by DNA damage with increased levels of p53 and ataxia telangiectasia mutated (ATM) (Kastan et al., 1991; Matsuoka et al., 2000) with repair of DNA damage before entry into the S-phase. Arrest in G₂ is caused by the inhibition of the cell division cycle control proteins prevent damaged chromosomes from entering the M-phase (Peng et al., 1997).

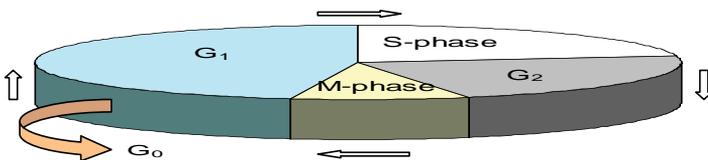


Figure 4. The cell cycle

Treatment

Surgery

Surgery is known to be an efficient treatment for CRC patients, where the skills of the surgeon are the most important factor in patient's outcome. In order to reduce postoperative complications, decrease the local recurrence rate and increase the overall survival, different surgical principles have to be followed. The surgeon has to have good resection margins (a tumour free margin of 10 cm on each side is recommended), perform lymph node clearance and sphincter preservation (Pålman, 1999). In patients with tumours located in the caecum, colon ascendans, hepatic flexure and the right side of the transverse colon a right sided hemicolectomy is performed. A left sided hemicolectomy may be used when the left side of transverse colon, flexura lienalis and the descending colon is extracted. A subtotal or total colectomy with ileorectal anastomosis might be considered if the patient has a synchronous tumour in both the right and left colon or in patients with HNPCC or FAP (Påhlman, 1999). Rectal surgery can be performed either by anterior resection or by a rectum amputation. A high anterior resection is performed when the tumour is placed around 11-15 cm distal from the anal verge. When the tumour is placed in the middle or distal part of the rectum an anterior resection with total mesorectal excision (TME) technique is performed, which means a sharp dissection with removal of the rectum and the mesorectum down to the pelvic floor, preserving nerves that regulate miction and potency (Heald et al., 1982). The TME technique was introduced early in the Scandinavian countries and is now considered as the golden standard in rectal cancer surgery. Abdomino-perineal rectum amputation means a total excision of rectum including the anal canal and the sphincter, and is performed when the tumour is placed close to anus (CRC Care Programs, 2008).

Radiotherapy

Radiation is charged electrons from high energetic photons that penetrates the tissue and causes DNA damage directly by ionisation within the DNA molecule or indirectly from the action of chemical radicals formed by local ionisation of water (Dizdaroglu, 1992). The general form of DNA damage are single strand breaks (SSBs) and double strand breaks (DSBs), where double strand breaks generally are lethal for the cell. Radiation can produce cell death by one of two mechanisms; apoptosis or necrosis (Figure 3), (Pawlik & Keyomarsi, 2004). Necrosis is an irreversible exogenous injury, with loss of membrane integrity, cell swelling, and dilation of cytoplasmic vesicles and random degradation of DNA (Kumar et al., 1997). Apoptosis is an active process characterized by programmed cell death in which a cascade of event is triggered in response to cellular stress (Kumar et al., 1997; Pawlik & Keyomarsi, 2004).

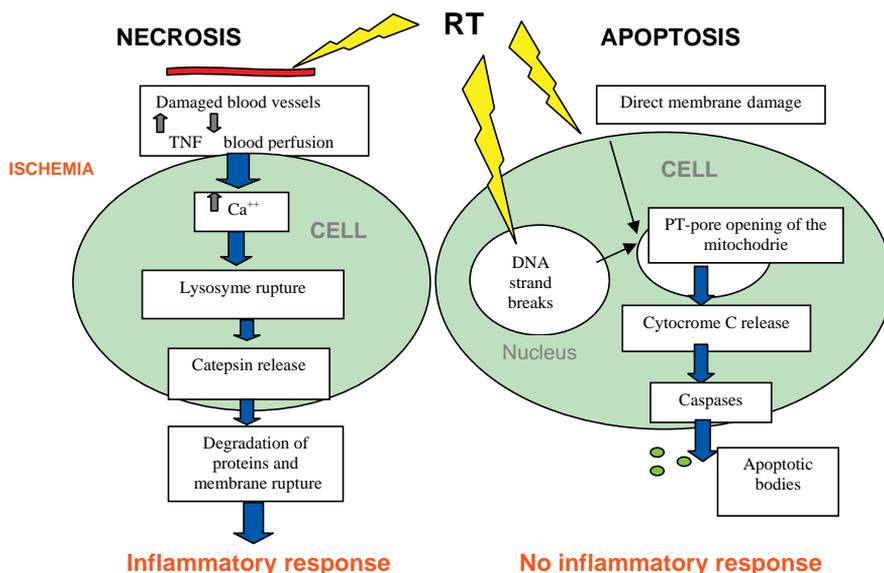


Figure 3. RT induced apoptosis and necrosis

RT can either be given as preoperative or postoperative treatment for primary resectable rectal tumours. The aim of RT is to eliminate microscopic peritumour cells in the peritumoural tissue and to gain a better local control. In the beginning of 1980, several randomised European studies investigated the effect of either short term (5 x 5Gy (Gray)) or long term preoperative RT (25 x 2Gy). In these studies, it was shown that both short term and long term preoperative RT reduced the local recurrence rate for rectal cancer patients (Cedermark et al., 1995; Pålman, 1997; Vermaas et al., 2005). At the same time, others showed that short term preoperative RT more efficiently reduced the local recurrence rate compared to long course postoperative RT (22% Vs13%), (Frykholm et al., 1993). The Swedish rectal cancer trial performed in 1987-1990, was the first study which showed that short term preoperative RT also improved the overall survival from 48% to 58% (Pålman, 1997). For this reason preoperative RT (5 X 5 Gy) is nowadays the golden standard for all primary resectable rectal tumours in Europe (Gerard et al.,1988), while in the United States postoperative RT is still frequent. The disadvantages of postoperative treatment are, risk of toxic damages to the small intestine (because the small intestine tend to fall down in the pelvis after surgery) and lower compliance due to postoperative complications that prolongs the RT start. The benefits of postoperative treatment are that you can select the best suited patients for RT after the histopathologic examination and you will reduce the risk of over-treating patients.

If the primary rectal tumour is not resectable, a long course preoperative RT treatment (up to 50.4 Gy) together with chemotherapy is usually given (2 cycles of oxaliplatin (Eloxatin)/capecitabine (Xeloda) before RT and capecitabine (Xeloda) alone during RT treatment), (CRC Care Programs, 2009).

The optimal clinical effect of RT is received approximately 5 weeks after RT, therefore an interval of one week between RT and surgery might be too short. In order to investigate at what time point after RT the optimal biological and clinical effect in tumour tissue is received, an ongoing Swedish rectal cancer trial (Stockholm III) randomises patients for either short course RT of 5 x 5 Gy, followed by surgery immediately or after 6-8 weeks, or with a long course RT with 2 x 25 Gy, followed by surgery within 6-8 weeks.

Recently, it was shown that short term preoperative RT was related to late complications such as increased mortality, reduced sphincter function, sexual dysfunction, and increased risk of postoperative ileus and other malignancies (Martling et al., 2001; Birgisson et al., 2005). These results further raised the question of whether preoperative RT has to be given more selective. In order to reduce the risk of late complications and to more selectively choose patients for preoperative RT, surgeons and oncologists in Europe nowadays divide rectal cancer patients into three subgroups called “good”, “bad” and “ugly”, where only the patients in the “bad” subgroup (stage T3b, N0/N1) receive short course preoperative RT (CRC Care Programs, 2008).

Chemotherapy and immunotherapy

In Sweden, the adjuvant chemotherapy treatment for CRC patients is based on the TNM classification system and patients performance status. In the Southeast Swedish Health Care region the adjuvant chemotherapy is given to CRC patients with a good performance status (WHO stage 0-2) and with radical excised stage II tumors with 2 or more risk factors (Table 2), or stage III tumors, no matter the risk factors. Rectal cancer patients with stage III disease plus 2 or more risk factors receives adjuvant chemotherapy. For younger patients (<71 years), 5-Fu based chemotherapy either administered intravenous or orally in combination with oxaliplatin (Eloxatin) is given. Older patients (>71 years) receive only 5-Fu based treatment. The adjuvant chemotherapy treatment is given for 6 months (CRC Care Programs, 2008, CRC Care Programs, 2009).

Table 2. Risk factors Vs non-risk factors for adjuvant CRC chemotherapy

Risk factors	Non-risk factors
Acute operation	No acute operation
MUCs	Non-MUCs
Poor differentiation	good, -moderate differentiation
Lymphovascular invasion	No lymphovascular invasion
Perineural growth	No perineural growth

The most common sites for local recurrence are the liver (50%), followed by the lung (25%), bone (10%) and brain (5%), (Eisenberg et al., 1982). Nowadays, patients with locally resectable liver and lung metastasis can be cured. The combination of neo-adjuvant chemother-

apy and surgery has dramatically improved the survival for patients with liver (30-40%) and lung (25%) metastasis (Giacchetti et al., 1999).

The aim of palliative chemotherapy is to improve patient's survival, quality of life and to prevent suffering. The main palliative treatment is based on 3 chemotherapies and 3 monoclonal antibodies, which could be given either alone or in combination. The first drug of choice is either 5-Fu/calciumfolinat (Leukovorin) or capecitabine (Xeloda) in combination with either oxaliplatin (Eloxatin) or irinotekan (Campto) (Ragnhammar et al., 2001).

Bevacizumab (Avastin) is a monoclonal antibody that inhibits angiogenesis by binding to the vascular endothelial growth factor (VEGF). It only works together with chemotherapy and in the Southeast Swedish Health Care region it is given with neo-adjuvant or palliative indication (CRC Care Programs, 2009). Cetuximab (Erbix) is a mouse-human chimeric monoclonal antibody which inhibits the epithelial growth factor receptor (EGFR), it is given in a palliative stage and can either be give alone (if no effect of oxaliplatin (Eloxatin) or irinotekan (Campto)) or in combination with chemotherapy (Ocvirk et al., 2010). The new EGFR inhibitor called panitumimab (Vectibix) is a fully human monoclonal antibody which has been demonstrated to have its clinical activity as a single agent in patients who had progressed on irinotecan (Campto), oxaliplatin (Eloxatin) or 5-Fu based therapies (Fakih & Wong, 2010; Keating, 2010). It has been proposed that panitumimab (Vectibix) can be used in patients with prior allergic reactions to cetuximab (Erbix) (Brugger, 2010).

Aims

The general aim of this study was to investigate the association of histological and biological factors with preoperative RT and clinical variables in rectal cancer patients who participated in a Swedish clinical rectal cancer trial of preoperative RT.

Specific aims

- To examine the relationships between survivin expression and preoperative RT, clinical or biological variables in rectal cancer patients.
- To analyse the association of PINCH expression with preoperative RT, clinical, histological and biological factors in rectal cancer patients, and to further study PINCH expression in relation to RT in fibroblast cell lines.
- To investigate the degree and location of LVD/BVD and their relationships to preoperative RT, clinical, histological and biologic factors in rectal cancer patients.
- To investigate the associations of inflammatory infiltration, fibrosis, necrosis and mucinous content, with preoperative RT, clinical and biological factors in rectal cancer patients.

Materials and Methods

Patients

In this thesis, information of patient's data such as gender, age, tumour location, stage, grade of differentiation, resection margin, surgical type and tumour number were obtained from surgical and pathological records. The clinical features and the number of tumour characteristics are presented in Table 3. Survival data were obtained from the Swedish National Board of Health and Welfare (Socialstyrelsen). The data of apoptosis performed by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling (TUNEL) assay (Adell et al., 2001) as well as the immunohistochemical staining for p53 (Adell et al., 1999), were taken from previous studies performed at our laboratory.

Papers I-IV, includes formalin-fixed paraffin embedded tissue from patients with primary rectal adenocarcinoma from the Southeast Swedish Health Care region who participated in a randomised clinical trial of preoperative RT between 1987-1990 (Påhlman, 1997). The patients were randomised to receive either surgery alone or preoperative RT and surgery. From the very beginning, a total of 171 patients were randomised, four patients were excluded and not surgically resected due to advanced disease and for four patients neither the surgical specimens nor the biopsies were obtained ($n = 163$). From the beginning of this thesis the mean follow up time was 63 months (range, 0-120 months). In the year of 2004, a new follow up study was performed which prolonged the mean follow up time to 86 months (papers II, III), (range, 0-193 months). At the same time the TNM staging system as well as the distance to the anal verge was included in our data (Table 3).

Forty-one patients (paper I), 65 (paper II), 64/66 (paper III) and 72 (paper IV) patients were randomly assigned to preoperative RT. These patients received a total dose of 25 Gy in 5 fractions over a median of 6 days (range, 5-12 days). The RT was administered by using a four-field technique from perineum to the promontory (L5-S1) extending laterally to the bony side walls of pelvis and from the middle of the caput femori to the anterior aspect of the sacrum. Risk organs were small intestine, anal-sphincter, nerves and urine bladder.

Surgery was then performed with a median of 3 days (range, 1-13 days) after RT. Fifty-seven (paper I), 72 (paper II), 74/74 (paper III) and 81 (paper IV) patients were randomised to surgery alone, respectively. None of the patients (paper I-IV) received chemotherapy before surgery. The number of the corresponding preoperative biopsies, distant normal mucosa and adjacent normal mucosa were studied in paper I, III, IV, as shown in Table 4. All the patients in this thesis have given their consent to participate in this study.

Table 3. The characteristics of patients and tumours

Characteristics	Non-RT/RT					
	Paper I <i>n</i> = 98	Paper II <i>n</i> = 137	Paper III <i>n</i> = 138/140	Paper IV <i>n</i> = 153		
Gender						
Male	36/22	42/40	43/43/40/41	46/42		
Female	21/19	30/25	31/31/24/25	35/30		
Age (years)		Age (years)		Age (years)		
≤67	20/14	≤67	30/26	31/30/28/29	≤66	29/28
>67	37/27	>67	42/39	43/44/36/37	>66	52/44
Dukes' stage		TNM		Dukes' stage		
A	19/14	I	20/22	19/20/21/22	A	22/21
B	14/15	IIA	18/21	19/19/20/20	B	22/24
C	22/8	IIIA	8/1	8/8/1/1	C	34/19
		IIIB	11/11	11/11/11/12		
		IIIC	11/4	12/11/4/4		
D	2/4	IV	4/6	5/5/7/7	D	3/8
Differentiation						
Good	2/3		2/2	5/5/5/5		2/2
Moderate	44/28		58/48	53/53/38/40		65/54
Poor	11/7		12/15	16/16/21/21		14/16
Unknown	0/3					
Surgical type						
Rectal amputation	31/17		36/25	37/37/25/27		44/30
Anterior resection	26/24		36/40	37/37/39/39		37/42
Resection margin						
Tumour free	56/38		70/61	72/72/59/61		78/67
Tumour	1/3		2/4	2/2/5/5		3/5
To anal verge (cm)						
Mean			7.5/8.5	8.5/7.6		7.3/8.3

Table 4. Numbers of the corresponding biopsies and distant/adjacent normal mucosa

Corresponding tissue	Paper I	Non-RT/RT	Paper IV
	<i>n</i>	Paper III <i>n</i>	<i>n</i>
Preoperative biopsies			78/75
Distant normal mucosa		18/17/20/11	
Adjacent normal mucosa	42/32	36/36/50/41	

Immunohistochemistry

Immunohistochemistry (IHC) is a method for detecting the presence of proteins in cells or tissues by using antibodies as specific reagents through antigen-antibody interactions. Antibodies used can be monoclonal or polyclonal. Monoclonal antibodies are generally considered to exhibit greater specificity, and polyclonal antibodies are a heterogeneous mix of antibodies that recognise several epitopes. There are two ways to detect antigens in tissue, via the direct or indirect method. The direct method is a one-step method and involves a labelled antibody reacting directly with the antigen in tissue sections (Figure 5a). In this thesis (papers I-III) the indirect staining method was used, which involves an unlabeled primary antibody that reacts with tissue antigen, and a labelled secondary antibody which reacts with the primary antibody as shown in figure 5b.

The immunohistochemical process was performed on 5µm section from paraffin blocks of surgical specimens from, distant normal mucosa (paper III), adjacent normal mucosa (papers I, III) and primary tumours (papers I-III). The sections were further deparaffinised and rehydrated with a series of decreasing concentrations of alcohol. To demask antigen epitopes, the sections were either boiled in a microwave oven (paper I), in a high pressure cooker (papers II, III) or in a calibration bath (paper III), (Julabo TW8). Endogenous peroxidase activity was inhibited with hydrogen peroxide and non-specific background staining was inhibited with serum-free protein block (Dako, Carpinteria, CA), the sections were incubated with a monoclonal primary antibody called survivin (paper I), (ab-1clone 8 E2, Neomarkers, Westinghouse, CA), a rabbit anti-PINCH antibody diluent in paper II (Dako) a mouse monoclonal D2-40 (prediluted; Abcam, Cambridge, UK) and CD34 antibody in paper III (18 µg/ml, Dako). The secondary antibody could be labelled with a fluorescent dye or an enzyme. In this thesis (papers I-III), the peroxidise-anti-peroxidase (PAP) technique was used where the secondary antibody was combined with an enzyme reagent of peroxidase-conjugated streptavidine. Further, a peroxidase substrate with 3,3-diaminobenzidine (DAB) was added for colour development (Figure 5). The section was counter stained and the result was investigated by light microscope.

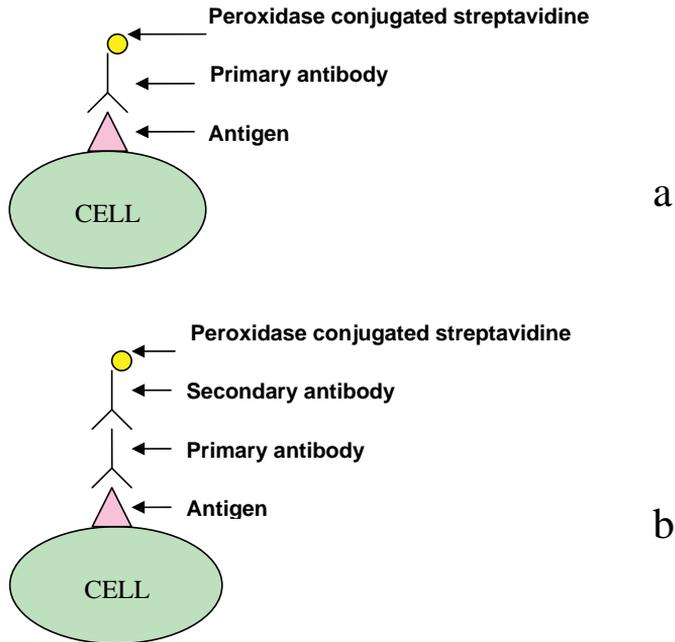


Figure 5. The direct (a) and indirect (b) immunohistochemical staining method

Cell line analysis and radiation procedure

In paper II, a cell line study was performed, where CCD-18 Co cell line derived from human colon fibroblasts (ATCC, Rockville, MD) were used. This was a kind gift from Dr. R Palmqvist from the Department of Pathology at the Umeå University of Sweden. The cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) with GlutamaxTM and supplemented with 1% Penicillin-Streptomycin and 10% Fetal Bovine Serum (FBS) (Invitrogen, Carlsbad, CA). The cells were grown in tissue culture flasks at 37°C with 5% CO₂ and passaged every few days to maintain exponential growth.

For all experiments, cells were seeded at a density of 60 000 cells/cm² and irradiated with photons from a 6MV linear accelerator Varian Clinac 600C/D (Varian Medical Systems, Palo Alto, CA). The field size was 30 x 30 cm and the distance between sources and cells was 100 cm. Acrylic glass plates were placed above (3 cm thick) and underneath (10 cm thick) the cells. The cells were exposed to single doses of 0, 2, 5 or 10 Gy at room temperature. The most significant biological change in protein expression was observed with the radiation dose of 2 Gy as also shown by previous studies (Farnebo et al., 2008). Therefore 2 Gy was used for further analysis. The controls (0Gy) were handled under the same environmental conditions as the treated cells. Following radiation, cells were harvested at 8, 24, 48 and 72h for western blot analysis.

Western blotting

In paper II, the western blot method was used, which is a method used in order to identify and quantify a certain protein in a cell lysate. This was performed by using gel-electrophoresis, followed by transfer of the separated proteins from the gel to a membrane, and subsequent immunoblotting of the protein of interest. Samples were prepared from tissue or cells that were homogenised in a buffer that protects the protein from degradation. The proteins were then separated by size through a Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), where the smaller proteins migrate through the gel faster than the bigger one. The proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane (paper II) for detection, and further incubated in fat-free dry milk in order to reduce the risk of non-specific protein interaction between the membrane and the primary antibody. The membrane was further incubated with a primary antibody that recognises the specific protein, and a secondary antibody, directed against the primary antibody. The secondary antibody was linked to a conjugate, as in paper II, an enzyme. Once the membrane has been incubated with the secondary antibody a detection substrate was added, which further reacted with the conjugate of the secondary antibody. The image of the antibodies bound to the blot was detected on photographic film (paper II). To quantitatively compare the loading of the wells the membranes were re-incubated with anti- β -actin antibody.

Haematoxylin and Eosin staining

The hematoxylin and eosin (HE) staining method was used in paper IV, where, 5 μ m thick paraffin-embedded sections were deparaffinised and hydrated, incubated in hematoxylin and rinsed with water and counterstained with eosin and then dehydrated. The staining was performed at the Department of Pathology at the University Hospital in Linköping.

Evaluation

The staining of IHC (papers I-III) and HE (paper IV) in biopsies, normal mucosa and tumours, were investigated by light microscope in 10–20 areas at x 400 magnification. The results were the mean of scores by two independent authors who had no knowledge about the patients or the postoperative histological examination.

In paper I, survivin expression was studied in primary tumours and adjacent normal mucosa. In primary tumours, the staining intensity was scored as; negative (no positive cells), equivocal (<5% positive cells), weak, or strong staining, for further analysis the negative and equivocal tumours were defined as a negative group and the weakly and strongly stained tumours as a positive group. The percentage of stained tumour cells was scored as <5%, 5–24%, 25–49%, 50–75%, or >75%, where the cutoff point was set to 25% (one group consisted of the cases with negative and <25 % of the stained tumor cells, and another group included the cases with \geq 25% of the stained tumor cells). In adjacent normal mucosa, the staining intensity

was scored as negative, equal, weaker, or stronger compared with the corresponding tumour cells.

In paper II, PINCH expression was analysed at the invasive margin and inner tumour area of primary tumours. The staining intensity was scored as negative, weak, moderate or strong staining, while the percentage of stained cells was scored as <25%, 25–49%, 50–75%, or >75%, respectively. Further the staining intensity was classified into a weak subgroup if they were negative, weak or moderately stained, or into a strongly stained subgroup if they were strongly stained. The expression of PINCH in CCD-Co 18 cells was quantified by western blot analysis and evaluated with the naked eye.

In paper III, LVD/BVD was assessed in distant normal mucosa, adjacent normal mucosa and primary tumours. The sections were investigated by using a double-headed light microscope, where the areas having the highest number capillaries and small venules were selected. Further, three hot spots were counted at x 200 magnification and the mean value of these three fields (hotspots) was used for further statistical analysis. By following a previous study by Kuokourakis et al (2000) the location of the hotspots was examined either in the periphery, the inner tumour area or invasive margin of the primary tumours. Two or three hotspots with the same location were combined and further analysed.

In paper IV, HE stained preoperative biopsies and primary tumours were examined. The primary tumours were analysed regarding inflammatory infiltration at the invasive margin and inner tumour area. No statistically significant difference of the inflammatory infiltration was found between the inner tumour area and invasive margin ($p = 0.88$), therefore for further analysis the results of the inflammatory infiltration at the inner tumour area and invasive margin were combined (paper IV, Table 2). The whole tumour specimen was further investigated for the content of fibrosis, necrosis and mucin. In biopsies, only inflammatory infiltration and necrosis were evaluated due to small tumour areas. A mean score was reached after examining 1-5 sections of each tumour/biopsy.

Statistical analysis

In papers I- IV, the chi-square method was used to test the differences in expression of survivin, PINCH, LVD/BVD location, inflammatory infiltration, fibrosis, necrosis and mucinous content in relation to clinical, pathological and biological factors. The McNemar method was used to test the differences in survivin expression between the adjacent normal mucosa and primary tumours (paper I) and the differences in inflammatory infiltration and necrosis between biopsies and tumours (paper IV).

In paper III, an independent and a dependent student's t-test were used to estimate the differences of LVD/BVD between normal mucosa and tumours. An independent student's t-test was used to test for the degree of LVD/BVD in tumours.

The expression of survivin, PINCH, LVD/BVD, inflammatory infiltration and necrosis in relation to survival of patients was tested by using Cox's Proportional Hazard Model by using both univariate and multivariate analyses. In paper II, a further interaction analysis was used to test the relationships between PINCH, RT and survival. Survival curves were calculated according to the Kaplan-Meier method (papers I-IV). Cox's logistic regression analyses were used to calculate odds ratio (OD) and confidence intervals (95%), (papers I, IV).

In all papers, the p-value was two sided and a p-value below 0.05 was considered significant.

Results

Paper I

In paper I, the expression of survivin was investigated in adjacent normal mucosa and primary tumours in relation to RT, clinical variables and biological factors (apoptosis and p53) in rectal cancer patients with or without RT.

Survivin expression was examined in 98 tumours, of which 32 (33%) had negative, 16 (16%) equivocal, 36 (37%) weak, and 14 (14%) had strong staining.

The relationship between survivin expression and clinical variables was analysed in all patients, and in the non-RT and RT subgroups. In all patients ($n = 98$), survivin-positive tumours were associated with worse survival compared to survivin-negative tumours ($p = 0.03$), (Paper I, Figure 2a). Even in a multivariate analysis the significance still remained adjusting for Dukes' stage, local and distant recurrence, grade of differentiation, gender, age, apoptosis and p53 ($p = 0.02$) (Paper I, Table 4). In patients without RT, survivin expression tended to be related to worse survival ($p = 0.08$), (Paper I, Figure 2b), while in patients with RT, survivin expression was not related to survival ($p = 0.19$), (Paper I, Figure 2c). There were no differences in survivin expression between the subgroups of non-RT and RT ($p = 0.71$).

The relationship between survivin expression and biological factors and RT showed that positive survivin compared to negative survivin tended to be related to less apoptosis in patients with RT ($p = 0.09$).

We further investigated survivin expression in adjacent normal mucosa in relation to RT. The survivin expression tended to increase after RT (from 33% to 67%) although the difference did not reach statistical significance ($p = 0.057$), (Paper I, Table 3).

Paper II

In paper II, the expression of PINCH and its relationship to RT, clinical variables, histological factors (lymphangiogenesis, angiogenesis, inflammatory infiltration and necrosis) and biological factors (apoptosis) were studied in rectal cancer patients with or without RT.

The expression of PINCH in primary tumours and its relationship to clinical variables were studied at the invasive margin and inner tumour area (Paper II, Figure 1a-b). At the invasive margin, either in all patients ($p = 0.04$) or in the non-RT subgroup ($p = 0.03$), (Paper II, Figure 2a), the patients with tumours having strong PINCH expression were related to worse survival, compared to the patients with tumours having weak PINCH expression. In the non-RT subgroup, the prognostic significance still remained after adjusting for TNM stage and differentiation ($p = 0.03$). No significant difference was found between PINCH expression and survival in the patients with RT ($p = 0.64$), (Paper II, Figure 2b). A further interaction analysis

between PINCH, RT and survival showed no significant difference ($p = 0.30$). There was no difference in PINCH expression between the non-RT and RT subgroups either at the invasive margin ($p = 0.68$) or at the inner tumour area ($p = 0.49$), (Table 2, Paper II).

We further analysed the PINCH expression at the invasive margin/inner tumour area and their relationship to pathological and biological factors. At the invasive margin, in all patients, strong PINCH expression was related to weak inflammatory infiltration ($p = 0.002$). In the non-RT group, strong PINCH expression was related to weak inflammatory infiltration ($p = 0.003$) and less apoptosis ($p = 0.02$). No significant relationships were found in the RT group ($p > 0.05$). At the inner tumour area, strong PINCH expression was related to weak inflammatory infiltration ($p = 0.0005$, $p = 0.0007$) in all patients and in the non-RT subgroup. In the RT group, strong PINCH expression was related to a higher grade of LVD ($p = 0.01$), (Paper II, Figure 3).

In the fibroblast cell line, there was no difference observed in PINCH expression between cells without RT (0Gy) or with RT (2Gy) harvested at different times (Paper II, Figure 4).

Paper III

In paper III, the relationship of the degree and location of LVD/BVD with RT, clinical variables, histological factors (inflammatory infiltration and fibrosis) and biological factors (apoptosis and p53) were studied in rectal cancer patients with or without preoperative RT.

The frequency of LVD was analysed at the periphery, the inner tumour area and the invasive margin of 138 primary tumours. Eighteen percent ($n = 25$) of the hotspots were found at the periphery, 52% ($n = 72$) at the inner tumour area and 22% ($n = 30$) at the invasive margin, 8% ($n = 11$) of the cases were negative for D2-40, (paper III, Figure 1).

The frequency of BVD was analysed at the periphery, the inner tumour area and the invasive margin in 140 primary tumours, where 63% of the cases were located at the periphery of the tumor, 34% ($n = 47$) at the inner tumour area and 3% ($n = 5$) at the invasive margin (Paper III, Figure 4), there were no negative cases for BVD.

The location of LVD and BVD expression was further analysed in relation to clinical, histological and biologic factors. In all patients, a higher LVD at the periphery was related to better survival compared to the inner tumour area/invasive margin ($p = 0.03$), (Paper III, Figure 2). Even in a multivariate analysis, the prognostic significance still remained independent of TNM stage, grade of differentiation and p53 ($p = 0.03$). LVD at the periphery had a higher rate of negative p53 expression compared to the inner tumour area/invasive margin ($p = 0.04$), (Paper III, Figure 3). No significance was found between LVD location and survival in the subgroups of non-RT ($p = 0.30$) and RT ($p = 0.50$). A high BVD at the periphery was related to worse survival in all patients ($p = 0.02$) and in patients without RT ($p = 0.007$), (Paper III, Figure 5). In patients without RT, adjustment for TNM stage and differentiation grade showed

a trend towards significance ($p = 0.05$). There was no significant relationship between BVD and survival in patients with RT ($p = 0.65$).

Paper IV

The relationship between the histological factors, inflammatory infiltration, fibrosis, necrosis and mucinous content and their association to RT, clinical variables, biological factors (p53, apoptosis and Cox-2) were studied in preoperative biopsies and primary tumours in rectal cancer patients with or without RT.

Inflammatory infiltration, fibrosis, necrosis and mucinous content were examined in the surgical specimens ($n = 148$), (paper IV, Figure 1a-d) and inflammatory infiltration and necrosis were evaluated in preoperative biopsies ($n = 153$).

The association between the histological factors and the clinical variables were studied in all patients as well as in the subgroups of non-RT and RT. In all patients, a higher grade of inflammatory infiltration was related to improved survival compared to weak inflammatory infiltration ($p = 0.004$), (Paper IV, Figure 2a). The prognostic significance still remained even in a multivariate analysis adjusting for gender, age, Dukes' stage, grade of differentiation, necrosis and fibrosis ($p = 0.01$), (Paper IV, Table 3). A higher grade of inflammatory infiltration was related to reduced distant recurrence rate ($p = 0.005$). In the non-RT group, a higher grade of inflammatory infiltration was related to improved survival ($p = 0.01$), (Paper IV, Figure 2b) and reduced distant recurrence rate ($p = 0.007$). In the RT group, a higher grade of inflammatory infiltration ($p = 0.04$), (Paper IV, Figure 2c) and strong necrosis ($p = 0.046$), (Paper IV, Figure 2d) were related to favourable survival.

Further, the relationship between the histological parameters and biological factors were investigated based on RT. When the non-RT and RT subgroup was compared, in all patients, the inflammatory infiltration in tumours was decreased ($p = 0.0003$), (Paper IV, Figure 3) and necrosis in tumours was increased ($p = 0.006$), (Paper IV, Figure 4). In tumors with negative p53 expression, tumours with RT, compared to tumours without RT, tended to have more necrosis (95% of 22 cases Vs 77% of 47, $p = 0.054$) and fibrosis (93% of 28 cases Vs 76% of 41, $p = 0.06$).

Discussion

In this thesis, we have shown that survivin expression, LVD location, PINCH expression at the invasive margin and inflammatory infiltration are independent prognostic factors in rectal cancer patients who participated in a clinical trial of preoperative RT.

In paper I, patients with positive survivin expression were related to worse survival compared to patients with negative survivin expression, in all patients, but not in the subgroups of non-RT and RT. The same relationship was found by others who studied rectal cancer patients treated with either the combination of radio-chemotherapy (Rödel et al., 2002) or did not receive any pre- or postoperative RT treatment (Kawasaki et al., 1998; Sarela et al., 2001). The relationship between positive survivin expression and worse survival in tumours might be explained by a survivin induced inhibition of caspase-3 and -7 which leads to reduced apoptosis and increased survival of the cell (Shin et al., 2001).

We also showed that positive survivin expression tended to be related to less apoptosis in tumours with RT, which was in line with several recent studies, where survivin was shown to be up-regulated and resistant to RT (Asanuma et al., 2000; Rödel et al., 2002; Rödel et al., 2003; Lu et al., 2004; Rödel et al., 2005). In recent years, survivin has been shown to play an important role in tumour development due to its regulation of apoptosis, proliferation and angiogenesis of the tumour tissue (Ambrosini et al., 1997; Adida et al., 1998; Sakao et al., 2005). Therefore, survivin nowadays attracts attention as a new target for anti-cancer therapy (Kanwar et al., 2001; Tu et al., 2003; Sharma et al., 2005; Kojima et al., 2006; Fisker et al., 2007; Ryan et al., 2009). In animal models, inactivation of survivin with AO has been shown to inhibit tumour growth (Kanwar et al., 2001; Tu et al., 2003). Furthermore, AO-mediated down-regulation of survivin in cancer cells was shown to enhance sensitivity to several chemotherapeutic drugs (Sharma et al., 2005; Kojima et al., 2006; Fisker et al., 2007) and has also been reported to increase sensitivity to RT (Sah et al., 2006). Right now several ongoing clinical trials further investigate the role of AO in cancer treatment.

The lymphatic vasculature drains interstitial fluid from tissue and is one of the most common ways for tumour cells to metastasise and spread. Few have investigated the location of LVD and its relationship to clinical outcome (Maula et al., 2003; Bono et al., 2004). In paper III, we showed that patients with LVD located at the periphery were related to better survival compared to patients with LVD at the inner tumour area/invasive margin, in all patients, but not in the subgroups of non-RT and RT. The positive relationship between LVD at the periphery and survival might be caused by a lower malignancy grade of tumour cells at the periphery compared to the inner tumour area/invasive margin where fewer metastases occurs (Palmqvist et al., 1998; Padera et al., 2002; He et al., 2005).

p53 is an important factor in the regulation of DNA repair, cell cycle and programmed cell death. In earlier studies of ours, on the same series of cases, it was shown that patients with negative (wild type) p53 expression were related to less local recurrence compared to patients with positive p53 expression (Adell et al., 2001). In our study, in all patients, LVD located at the periphery was related to negative p53 expression compared to the inner tumour area/invasive margin. This strengthens our previous positive relationship between LVD location and survival, and makes us suggest that the tumour cells and their surrounding lymphatic vessels at the periphery might create an environment that makes it difficult for malignant cells to transit into the lymphatic circulation.

PINCH is known to be critically involved in cell cycle progression and survival (Guo & Wu., 2002; Eke et al., 2010) and related to a worse outcome in CRC patients (Gao et al., 2004). In line with previous results, we showed that strong PINCH expression at the invasive margin of tumours was related to worse survival compared to weak PINCH expression. There was no significant relationship between PINCH and survival in the RT group. A further interaction analysis between PINCH, RT and survival showed no statistically significant difference, which might be explained by too few deaths in the RT subgroup. As far as we know, this is the first study that analyses the relationship between PINCH and RT in patients. A previous study showed that PINCH enhanced radio-resistance by inhibiting PP1 α via the Akt-1 pathway in cell lines (Eke et al., 2010). Others showed that EGF was upregulated by RT (Schmidt-Ullrich et al., 1994; Ruifrok et al., 1997) and associated with PINCH via the adaptor protein Nck-2 (Tu et al., 1998). The differences in survival for patients with PINCH expression with and without RT, and the lack of changes in PINCH expression with or without RT, makes us suggest that RT does not seem to directly affect PINCH expression, but PINCH might be activated by RT through some other biological pathways.

In tumours with RT, we found a positive relationship between PINCH at the inner tumour area and LVD. Since PINCH is involved in the proliferation of cells, PINCH might stimulate the production of new lymph vessels as a reaction to RT induced cell damage. The positive relationship between PINCH and LVD in tumours with RT might increase the area for potential escape of tumour cells into the lymphatic circulation.

In this study, strong PINCH expression was also related to weak inflammatory infiltration both at the inner tumour area and invasive margin, in all patients as well as in the non-RT subgroup. In line with our findings, Gao et al (2005) showed that PINCH was increased in myofibroblasts, suggesting that these cells induced the tumour reaction against inflammatory cells. We also found an association between strong PINCH expression at the invasive margin and less apoptosis in the non-RT group. These findings suggest that PINCH at the invasive margin might facilitate tumour progression and survival by inhibiting inflammatory infiltration and reduce apoptosis.

A better understanding of the interplay between tumours and their immunologic microenvironment is of great importance for the development of prognostic markers and therapeutic strategies. In paper IV, we showed that patients with strong inflammatory infiltration were related to a better outcome compared to patients with weak inflammatory infiltration in all patients as well as in the subgroups of non-RT and RT. The presence of inflammatory infiltration was also associated with decreased rate of distant recurrence, in all patients, and in the non-RT group, but not in the RT group. This was in line with other studies of rectal and CRC patients where no subgroup analysis of non-RT and RT patients was performed (Nagtegaal et al., 2001) or no RT or chemotherapy was given (Gao et al., 2005; Klintrup et al., 2005). The underlying mechanism of infiltration in relation to prognosis is suggested to be caused by the activation of inflammatory cells with a final lysis of the tumour cell (Arancia et al., 1990; Barth et al., 1996). Since most human tumours are associated with a diverse immune cell infiltration, many researchers have put a lot of effort on trying to score what type of inflammatory cells that infiltrate the tumours, and further investigate how they are related to clinical parameters. In a previous study performed at our laboratory, it was shown that the most common immune cells were T-lymphocytes in Dukes' stage A+ B CRC tumors (Håkansson et al., 1997). Recently, it was shown that a high level of infiltrating T-cells was a strong predictor for reduced recurrence and improved survival in CRC patients (Camus & Galon, 2010; Fridman et al., 2010; Simpson et al., 2010), therefore, for the future, an immune score might be beneficial to predict recurrence and survival in early stage CRC tumours (Fridman et al., 2010).

MVD and its relationship to survival have been studied extensively (Kuokourakis et al., 2000; Miyata et al., 2006), but very few have investigated the relationship of BVD with RT and survival. One previous author showed that a decrease in MVD after RT was related to improved survival (Lövey et al., 2006). In line with previous findings, our study showed that a high BVD at the periphery was related to worse survival in all patients and in patients without RT. In patients without RT, a further multivariate analysis showed a trend towards significance, but no significant relationship was found in patients with RT. The tendency of increased survival for patients with BVD at the periphery might be caused by an RT induced destruction of blood vessels which reduces the surface area for potential escape of tumour cells into the blood circulation (Baeten et al., 2006). This issue needs to be addressed further on a larger series of patients.

Necrosis has been associated with a poor clinical outcome in patients with CRC (Swinson et al., 2002; Gao et al., 2005). In contrast to the previous findings, our study showed that necrosis was increased by RT and related to better survival. The relationship between RT induced necrosis and survival might be caused by an RT induced destruction of blood vessels that reduces the risk for tumour cells to spread hematogenously. There seems to be two ways to initiate necrosis, by rapid tumour growth and by irradiation.

A previous study on the same series of cases showed that the local recurrence rate was reduced in p53 negative (wild type) tumours compared to p53 positive tumours after RT (Adell et al., 2001). Here, necrosis and fibrosis tended to increase after RT in p53 negative tumours compared to p53 positive tumours. It was shown that fibrosis was related to favourable survival and better differentiation in rectal cancer patients without preoperative RT or chemotherapy (Ueno et al., 2004) and that RT induced necrosis was related to a better outcome in rectal cancer patients. The explanation for the relationship between p53, RT, fibrosis and necrosis could be an RT induced production of growth factors that stimulates the myofibroblasts to produce fibrosis (Martin et al., 2000). RT induced necrosis might be initiated by destruction of capillaries which leads to oxygen deficiency in the tissue and necrosis. We propose that the patients with p53 negative tumours that had a better response to RT, might be via RT induced necrosis and fibrosis through some p53 pathway.

The inflammatory infiltration and its association to RT have been studied, but the relationship is still not yet clear. It was shown that low-dose RT used as a treatment for painful joint diseases had a pronounced anti-inflammatory effect; while on the contrary, the high dose of RT given in cancer therapy induced the expression of pro-inflammatory cytokines (Hallahan et al., 1996; Hildebrandt et al., 1998). Here, we and others (Nagtegaal et al., 2002) observed that the infiltration was immediately decreased after RT, which might be explained by a first rapid reaction to RT with decreased numbers of immune cells and suppressed immune cell function (Hildebrandt et al., 1998; Ebert, 1999, Martin et al., 2000), further, one week after RT the immune cells were repopulating (Nagtegaal et al., 2002). High dose RT seems to immediately depress the inflammatory infiltration, but after a while the immune cells seems to repopulate.

Conclusions

Survivin, PINCH, LVD and inflammatory infiltration are independent prognostic factors in rectal cancer patients either in the whole group of patients or in the subgroups of non-RT or RT. RT does not seem to directly affect PINCH expression but PINCH might be activated by RT via other biological pathways. The positive relationship between PINCH and LVD in tumours after RT might increase the area for potential escape of tumour cells into the lymphatic circulation. The patients with p53 negative tumours that had a better response to RT might be affected via RT induced necrosis and fibrosis through some p53 pathway. The findings may help us to improve patient's survival by selecting the best suited patients for preoperative RT in the future.

Acknowledgements

During my graduate study years I have had the pleasure to meet and work with many supportive and friendly people. I would like to express my gratitude to everybody who has contributed in some way to this thesis; especially I would like to thank:

All the rectal cancer patients who gave their consent to donate their surgical material for scientific research

The Swedish Cancer Foundation, Swedish Research Council and the Health Research Council in the South-East of Sweden, Rotarys cancer stiftelse, Öland, for financial support

Xiao-Feng Sun, my supervisor and friend. Thank you for always being glad and friendly. During all these years you have motivated and inspired me, you have always believed in me and you have made me grow as a researcher

Gunnar Adell my co-supervisor, for collecting all the material and clinical data used in this thesis. Thanks for interesting discussions and for putting in clinical aspects into my research

Bo Nordenskjöld for your great support during my time as a researcher and for your strong belief in me and my abilities

Birgitta Holmlund for dear friendship, brilliant collaboration, laborative knowledge and inspiring discussions

Jingfang Gao for dear friendship, perfect collaboration and stimulating discussions

John Carstensen for interesting statistical discussions and good statistical advices

Tomas Walz for making it possible for me to work clinically and with research

Birgit Olsson for your friendly attitude, guidance and technical advice at the laboratory

Åsa Wallin, Agneta Jansson, Daniella Pfeifer, Andreas Levander, Marie Ahnström Waltersson, Karin Söderlund Leifler, Josefine Bostner, Sebastian Gnosa, Johannes Straatman. Thanks for all good laborative advices and for technical assistance and for putting up with me and my sometimes “silly” questions

David Hiselwood for your kindness and help regarding the revision of my thesis

Maria Albertsson for stimulating clinical discussions and for helping me with the revision of my thesis

Kerstin Nordenskjöld for your kindness and support during my time at the Oncology center in Linköping

Chatarina Malm for your kindness and help with the administrative work

All my colleagues at the Oncology department and KEF for making a friendly and inspiring work atmosphere

Lena Wigren for your friendly attitude and for all help with the administration

Anette Cederberg for your kindness and technical support

Ann-Charlotte Johansson and **Karin Roberg** for sharing all your knowledge about cell culture and radiation procedures

Rikard Palmqvist and **Maria Henriksson** for providing us with the cell lines used in this thesis

Dan Josefsson and **Sara Olsson** for helping me with the radiation of the cells

My dear husband **Rikard** and my two princesses **Frida** and **Sofia**, for your endless love and for always being there when life goes up and down

My father **Olav Knutsen** for his dear friendship and great support during all these years and for raising me to become the person I am today

My mother **Inger Knutsen** for your love and support during my childhood and for raising me to become an independent person

To my husbands parents **Ingvor** and **Evert Holmqvist** for your love and for all your support to me and my family

My brother and sisters and my husband's sister and families for your dear and close friendship

To my friend **Camilla Broman** for helping me revising the Swedish summary of the thesis

My cousin and close friend **Jessica Skarph** who passed away all too early, you are one of the reasons for why I wanted to become an oncologist

References

- Aarnio M, Sankila R, Pukkala E et al. Cancer risk in mutation carriers of DNA-mismatch-repair genes. *Int J Cancer* 1999;81:214-18.
- Adachi Y, Mori M, Kuroiwa S et al. Histopathologic evaluation of survival time in patients with colorectal carcinoma. *J Surg Oncol* 1989;42:219-24.
- Adell GC, Sun X-F, Stål O et al. p53 status: An indicator for the effect of preoperative radiotherapy in rectal cancer. *Radiother Oncol* 1999;51:169-74.
- Adell GC, Zang H, Evertsson S et al. Apoptosis in rectal cancer: Prognosis and recurrence after preoperative radiotherapy. *Cancer* 2001;91:1870-75.
- Adida C, Berrebi D, Peuchmaur M et al. Anti-apoptosis gene, survivin, and prognosis of neuroblastoma. *Lancet* 1998;351:882-83.
- Agassy-Cahalon L, Yaakubowicz M, Witz IP et al. The immune system during the precancer period: naturally-occurring tumor reactive monoclonal antibodies and urethane carcinogenesis. *Immunol Lett* 1988;18:181-89.
- Aki T, Mizukami Y, Oka Y et al. Phosphoinositide 3-kinase accelerates necrotic cell death during hypoxia. *Biochem J* 2001;358:481-87.
- Akino F, Mitomi H, Nakamura T et al. High apoptotic activity and low epithelial cell proliferation with underexpression of p21^{WAF1/CIP1} and p27^{Kip1} of mucinous carcinomas of the colorectum. *Am J Clin Pathol* 2002;117:908-15.
- Alitalo K, Tammela T, Petrova TV. Lymphangiogenesis in development and human disease. *Nature* 2005;438:946-53.
- Altieri DC. Targeted therapy by disabling crossroad signaling networks: the survivin paradigm. *Mol Cancer Ther* 2006;5:478-82.
- Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat Med* 1997;3:917-21.
- Anguiano-Hernandez Y, Chartier A, Huerta S. Smac/DIABLO and colon cancer. *Anticancer Agents Med Chem* 2007;7:467-73.

- Arancia G, Malorni W, Donelli G. Cellular mechanisms of lymphocyte-mediated lysis of tumor cells. *Ann Ist Super Sanita* 1990;26:369-84.
- Arora PD, McCulloch CA. The Deletion of Transforming Growth Factor- β -Induced Myofibroblasts Depends on Growth Conditions and Actin Organization. *Am J Pathol* 1999;155:2087-99.
- Asanuma K, Moriai R, Yajima T et al. Survivin as a radioresistance factor in pancreatic cancer. *Jpn J Cancer Res* 2000;91:1204-09.
- Badoual C, Hans S, Rodriguez J et al. Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers. *Clin Cancer Res* 2006;12:465-72.
- Baeten CI, Castermans K, Lammering G et al. Effects of radiotherapy and chemotherapy on angiogenesis and leukocyte infiltration in rectal cancer. *Int J Radiat Oncol Biol Phys* 2006;66:1219-27.
- Barth RJ, Camp BJ, Martuscello TA et al. The cytokine microenvironment of human colon carcinoma. Lymphocyte expression of tumor necrosis factor- α and interleukin-4 predicts improved survival. *Cancer* 1996;78:1168-78.
- Bergman PJ, Harris D. Radioresistance, chemoresistance, and apoptosis resistance. The past, present, and future. *Vet Clin North Am Small Anim Pract* 1997;27:47-57.
- Bernstein H, Bernstein C, Payne CM et al. Bile acids as endogenous etiologic agents in gastrointestinal cancer." *World J Gastroenterol* 2009;15:3329-40.
- Birgisson H, Pahlman L, Gunnarsson U et al. Adverse effects of preoperative radiation therapy for rectal cancer: long-term follow-up of the Swedish Rectal Cancer Trial. *J Clin Oncol* 2005; 23:8697-8705.
- Bono P, Wasenius VM, Heikkilä P et al. High LYVE-1-positive lymphatic vessel numbers are associated with poor outcome in breast cancer. *Clin Cancer Res* 2004;10:7144-49.
- Bosman FT. Prognostic value of pathological characteristics of colorectal cancer. *Eur J Cancer* 1995;31A:1216-21.
- Boyle P, Leon ME. Epidemiology of colorectal cancer. *Br Med Bull* 2002;64:1-25.
- Brigati CD, Noonan, Albini A et al. Tumors and inflammatory infiltrates: Friends or foes? *Clin Exp Metastasis* 2002;19:247-58.

- Brodt P, Gordon J. Natural resistance mechanisms may play a role in protection against chemical carcinogenesis. *Cancer Immunol Immunother* 1982;13:125-27.
- Brugger W. Successful treatment with the fully human antibody panitumumab after a severe infusion reaction with cetuximab. *Tumori* 2010;96:473-77.
- Brändén H. *Grundläggande immunologi*. Lund, Studentlitteratur, Sweden 1995.
- Camus M, Galon J. Memory T-cell responses and survival in human cancer: remember to stay alive. *Adv Exp Med Biol* 2010;684:166-77.
- Care Programs for Colorectal Oncology in the Southeast Health Care Region. Sweden 2009.
- Cedermark B, Johansson H, Rutqvist LE et al. The Stockholm I trial of preoperative short term radiotherapy in operable rectal carcinoma. A prospective randomized trial. Stockholm Colorectal Cancer Study Group. *Cancer* 1995;75:2269-75.
- Chang F, Syrjänen S, Syrjänen K et al. Implications of the p53 tumor-suppressor gene in clinical oncology. *J Clin Oncol* 1995;13:1009-22.
- Chen K, Tu, Y, Zhang Y et al. PINCH-1 regulates the ERK-Bim pathway and contributes to apoptosis resistance in cancer cells. *J Biol Chem* 2008;283:2508-17.
- Coca S, Perez-Piqueras J, Martinez D et al. The prognostic significance of intratumoral natural killer cells in patients with colorectal carcinoma. *Cancer* 1997;79:2320-28.
- Coussens, LM, Werb Z. Inflammation and cancer. *Nature* 2002;420:860-67.
- Cunningham J, Lust JA, Schaid DJ et al. Expression of p53 and 17p Allelic Loss in Colorectal Carcinoma. *Cancer Res* 1992;52: 1974-80.
- Daniel D, Chiu C, Giraudo E et al. CD4+ T cell-mediated antigen-specific immunotherapy in a mouse model of cervical cancer. *Cancer Res* 2005;65:2018-25.
- Deans GT, Patterson CC, Parks TG et al. Colorectal carcinoma: importance of clinical and pathological factors in survival. *Ann R Coll Surg Engl* 1994;76: 59-64.
- de Bruin EC, Medema JP. Apoptosis and non-apoptotic deaths in cancer development and treatment response. *Cancer Treat Rev* 2008;34:737-49.
- Des Guetz, Uzzan B, Nicolas P et al. Microvessel density and VEGF expression are prognostic factors in colorectal cancer. Meta-analysis of the literature. *Br J Cancer* 2006;94:1823-32.

- de Visser K, Eichten A, Coussens LM et al. Paradoxical roles of the immune system during cancer development. *Nat Rev Cancer* 2006;6:24-37.
- Dizdaroglu, M. Measurement of radiation-induced damage to DNA at the molecular level. *Int J Radiat Biol* 1992;61:175-83.
- Dolcetti R, Viel A, Doglioni C et al. High prevalence of activated intraepithelial cytotoxic T lymphocytes and increased neoplastic cell apoptosis in colorectal carcinomas with microsatellite instability. *Am J Pathol* 1999;154:1805-13.
- Dougherty G, Jose C, Gimona M et al. The Rsu-1-PINCH1-ILK complex is regulated by ras activation in tumor cells. *Eur J Cell Biol* 2008;87:721-34.
- Downward J. Ras signalling and apoptosis. *Curr Opin Genet Dev* 1998;8:49-54.
- Dvorak HF, Brown LF, Detmar M et al. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* 1995;146:1029-39.
- Eberhart CE, Coffey RJ, Radhika A et al. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* 1994;107:1183-88.
- Ebert EC. Inhibitory effects of transforming growth factor-beta (TGF-beta) on certain functions of intraepithelial lymphocytes. *Clin Exp Immunol* 1999;115:415-20.
- Eisenberg B, Decosse JJ, Harford F et al. Carcinoma of the colon and rectum: the natural history review of 1704 patients. *Cancer* 1982;49:1131-34.
- Eke I, Koch U, Hehlhans S et al. PINCH1 regulates Akt1 activation and enhances radioresistance by inhibiting PP1alpha. *J Clin Invest* 2010;120:2516-27.
- Emmrich, J, Weber I, Nausch M et al. Immunohistochemical characterization of the pancreatic cellular infiltrate in normal pancreas, chronic pancreatitis and pancreatic carcinoma. *Digestion* 1998;59:192-98.
- Fakih M, Wong R. Efficacy of the monoclonal antibody EGFR inhibitors for the treatment of metastatic colorectal cancer. *Curr Oncol* 2010;17:S3-S17.
- Farnebo L, Jerhammar F, Vainikka L et al. Number of negative points: a novel method for predicting radiosensitivity in head and neck tumor cell lines. *Oncol Rep* 2008;20:453-61.

- Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990;61:759-767.
- Fei P, Bernhard EJ, et al. Tissue-specific Induction of p53 Targets in Vivo. *Cancer Res* 2002;62:7316-27.
- Fernig, D, Gallagher G. Fibroblast growth factors and their receptors: an information network controlling tissue growth, morphogenesis and repair. *Prog Growth Factor Res* 1994;5:353-77.
- Fisker N, Westergaard M, Hansen HF et al. Survivin mRNA antagonists using locked nucleic acid, potential for molecular cancer therapy. *Nucleosides Nucleotides Nucleic Acids* 2007;26:1427-30.
- Foght F, Zimmerman RL, Ross HM et al. Identification of lymphatic vessels in malignant, adenomatous and normal colonic mucosa using the novel immunostain D2-40. *Oncol Rep* 2004;11:47-50.
- Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med* 1971;285:1182-86.
- Folkman J. Role of angiogenesis in tumor growth and metastasis. *Semin Oncol* 2002;29:15-18.
- Fox SB, Gasparini G, Harris AL et al. Angiogenesis: pathological, prognostic, and growth-factor pathways and their link to trial design and anticancer drugs. *Lancet Oncol* 2001;2:278-89.
- Franceschi S, Dal Maso L, Augustin L et al. Dietary glyceemic load and colorectal cancer risk. *Ann Oncol* 2001;12:173-78.
- Freeman, HJ. Dietary fibre and colonic neoplasia. *Can Med Assoc J* 1979;121:291-296.
- Fridman WH, Galon J, Dieu-Nosjean MC et al. Immune Infiltration in Human Cancer: Prognostic Significance and Disease Control. *Curr Top Microbiol Immunol* 2010 [Epub ahead of print].
- Frykholm GJ, Glimelius B, Pählman L. Preoperative or postoperative irradiation in adenocarcinoma of the rectum: final treatment results of a randomized trial and an evaluation of late secondary effects. *Dis Colon Rectum* 1993;36:564-72.

- Fu SL, Wu YL, Zhang YP et al. Anti-cancer effects of COX-2 inhibitors and their correlation with angiogenesis and invasion in gastric cancer. *World J Gastroenterol* 2004;10:1971-74.
- Fukuda T, Chen K, Shi X et al. PINCH-1 is an obligate partner of integrin-linked kinase (ILK) functioning in cell shape modulation, motility, and survival. *J Biol Chem* 2003;278:51324-333.
- Gabbiani G, Ryan GB, Majne G. Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. *Experientia* 1971;27:549-50.
- Gao J, Arbman G, Rearden A et al. Stromal staining for PINCH is an independent prognostic indicator in colorectal cancer. *Neoplasia* 2004;6:796-801.
- Gao JF, Arbman G, Whadra TI et al. Relationships of tumor inflammatory infiltration and necrosis with microsatellite instability in colorectal cancers. *World J Gastroenterol* 2005;11:2179-83.
- Garcia-Ruiz C, Colell A, Mari M et al. Direct effect of ceramide on the mitochondrial electron transport chain leads to generation of reactive oxygen species. Role of mitochondrial glutathione. *J Biol Chem* 1997;272:11369-77.
- Gerard A, Buyse M, Nordlinger B et al. Preoperative radiotherapy as adjuvant treatment in rectal cancer. Final results of a randomized study of the European Organization for Research and Treatment of Cancer (EORTC). *Ann Surg* 1988;208:606-14.
- Giacchetti S, Itzhaki M, Gruia G et al. Long-term survival of patients with unresectable colorectal cancer liver metastases following infusional chemotherapy with 5-fluorouracil, leucovorin, oxaliplatin and surgery. *Ann Oncol* 1999;10:663-69.
- Gianani R, Jarboe E, Orlicky D et al. Expression of survivin in normal. Hyperplastic, and neoplastic colonic mucosa. *Hum Pathol* 2001;32:119-25.
- Glasgow SC, Yu J, Carvalho LP et al. Unfavourable expression of pharmacologic markers in mucinous colorectal cancer. *Br J Cancer* 2005;92:259-64.
- Gorski DH, Beckett MA, Jaskowiak NT et al. Blockage of the vascular endothelial growth factor stress response increases the antitumor effects of ionizing radiation. *Cancer Res* 1999;59:3374-78.
- Gravalos C, Garcia-Escobar I, Garcia-Alfonso P et al. Adjuvant chemotherapy for stages II, III and IV of colon cancer. *Clin Transl Oncol* 2009;11: 526-33.

- Green D, Reed J. Mitochondria and apoptosis. *Science* 1998;281:1309-12.
- Guo L, Wu C. Regulation of fibronectin matrix deposition and cell proliferation by the PINCH-ILK-CH-ILKBP complex. *FASEB J* 2002;16:1298-300.
- Half E, Bercovich D, Rozen P. Familial adenomatous polyposis. *Orphanet Journal of Rare Diseases* 2009;4:1-23.
- Hallahan D, Kuchibhotla J, Wyble C. Cell adhesion molecules mediate radiation-induced leukocyte adhesion to the vascular endothelium. *Cancer Res* 1996;56:5150-55.
- He Y, Rajantie I, Pajusola K et al. Vascular endothelial cell growth factor receptor 3-mediated activation of lymphatic endothelium is crucial for tumor cell entry and spread via lymphatic vessels. *Cancer Res* 2005;65:4739-46.
- Heald RJ, Husband EM, Ryall RD et al. The mesorectum in rectal cancer surgery--the clue to pelvic recurrence? *Br J Surg* 1982;69:613-16.
- Hildebrandt G, Seed MP, Freemantle CN et al. Mechanisms of the anti-inflammatory activity of low-dose radiation therapy. *Int J Radiat Biol* 1998;74:367-78.
- Hoebke K, Janssen E, Beutler B et al. The interface between innate and adaptive immunity. *Nat Immunol* 2004;5:971-74.
- Hoffman WH, Biade S, Zilfou JT et al. Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. *J Biol Chem* 2002;277:3247-57.
- Huang CG, Gopalakrishna GS, Nichols BL. Fiber, intestinal sterols, and colon cancer. *Am J Clin Nutr* 1978;31:516-526.
- Håkansson L, Adell, Boeryd B et al. Infiltration of mononuclear inflammatory cells into primary colorectal carcinomas: an immunohistological analysis. *Br J Cancer* 1997;75:374-80.
- Inoue A, Moriya H, Katada N et al. Intratumoral lymphangiogenesis of esophageal squamous cell carcinoma and relationship with regulatory factors and prognosis. *Pathol Int* 2008;58:611-19.
- Jackowski S, Janusch M, Fiedler E et al. Radiogenic lymphangiogenesis in the skin. *Am J Pathol* 2007;171:338-48.
- Joukov V, Kaipainen A, Jeltsch M et al. Vascular endothelial growth factors VEGF-B and VEGF-C. *J Cell Physiol* 1997;173:211-15.

- Juin P, Hueber AO, Littlewood T et al. c-Myc-induced sensitization to apoptosis is mediated through cytochrome c release. *Genes Dev* 1999;13:1367-81.
- Kanwar JR, Shen WP, Kanwar RK et al. Effects of survivin antagonists on growth of established tumors and B7-1 immunogene therapy. *J. Natl. Cancer Inst* 2001;93:1541-52.
- Kapiteijn E, Marijnen C, Nagtegaal ID et al. Preoperative Radiotherapy Combined with Total Mesorectal Excision for Resectable Rectal Cancer. *N Engl J Med* 2001;345:638-46.
- Kastan MB, Onyekwere O, Sidransky D et al. Participation of p53 protein in the cellular response to DNA damage. *Cancer Res* 1991;51:6304-11.
- Katsenelson NS, Shurin GV, Bykovskaia SN et al. Human Small Cell Lung Carcinoma and Carcinoid Tumor Regulate Dendritic Cell Maturation and Function. *Mod Pathol* 2000;14:40-5.
- Kawamori T, Rao CV, Seibert K et al. Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. *Cancer Res* 1998;58:409-12.
- Kawasaki H, Altieri DC, Lu CD et al. Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer. *Cancer Res* 1998;58:5071-74.
- Keating GM. Spotlight on panitumumab in metastatic colorectal cancer. *BioDrugs* 2010;24:275-78.
- Klintrup K, Mäkinen JM, Kauppila S et al. Inflammation and prognosis in colorectal cancer. *Eur J Cancer* 2005;41:2645-54.
- Knudson AG. Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A* 1971;68:820-23.
- Kojima HM, Iida M, Yaguchi Y et al. Enhancement of Cisplatin sensitivity in squamous cell carcinoma of the head and neck transfected with a survivin antisense gene. *Arch Otolaryngol Head Neck Surg* 2006;132:682-85.
- Koukourakis MI, Giatromanolaki A, Sivridis E et al. Cancer vascularization: implications in radiotherapy? *Int J Radiat Oncol Biol Phys* 2000;48:545-53.
- Kulaylat MN, Dayton MT. Ulcerative colitis and cancer. *J Surg Oncol* 2010;101:706-12.
- Kumar V, Cotran R, Robbins S. Basic pathology, 6th edition. Philadelphia, Pennsylvania, W.B Saunders Company 1997.

- Lei Y, Geng Z, Guo-Jun W et al. Prognostic significance of survivin expression in renal cell cancer and its correlation with radioresistance. *Mol Cell Biochem* 2010;344:23-31.
- Levine AJ. The tumor suppressor genes. *Annu Rev Biochem* 1993;62:623-51.
- Levine AJ, Perry ME, Chang A et al. The 1993 Walter Hubert Lecture: the role of the p53 tumour-suppressor gene in tumorigenesis. *Br J Cancer* 1994;69:409-16.
- Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat Rev Immunol* 2005;5:331-42.
- Loukola, A, Salovaara R, Kristo P et al. Microsatellite Instability in Adenomas as a Marker for Hereditary Nonpolyposis Colorectal Cancer. *Am J Pathol* 1999;155:1849-53.
- Lu B, Mu Y, Cao C et al. Survivin as a therapeutic target for radiation sensitization in lung cancer. *Cancer Res* 2004;64:2840-45.
- Lövey J, Lukits J, Remenar E et al. Antiangiogenic effects of radiotherapy but not initial microvessel density predict survival in inoperable oropharyngeal squamous cell carcinoma. *Strahlenther Onkol* 2006;182:149-56.
- Mao XW. A quantitative study of the effects of ionizing radiation on endothelial cells and capillary-like network formation. *Technol Cancer Res Treat* 2006;5:127-34.
- Mao XY, Wang XG, Lv XJ et al. COX-2 expression in gastric cancer and its relationship with angiogenesis using tissue microarray. *World J Gastroenterol* 2007;13:3466-71.
- Martin M, Lefaix JL, Delanian S. TGF- β 1 and radiation fibrosis: A master switch and a specific therapeutic target? *Int J Radiation Oncology Biol Phys* 2000;47:277-90.
- Martling A, Holm T, Johansson H et al. The Stockholm II trial on preoperative radiotherapy in rectal carcinoma: long-term follow-up of a population-based study. *Cancer* 2001;92:896-902.
- Matsuoka S, Rotman G, Ogawa A et al. Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro. *Proc Natl Acad Sci U S A* 2000;97:10389-94.
- Maula SM, Luukka M, Grenman R et al. Intratumoral lymphatics are essential for the metastatic spread and prognosis in squamous cell carcinomas of the head and neck region. *Cancer Res* 2003;63:1920-26.
- Mehrotra S, Languino LR, Raskett CM et al. IAP regulation of metastasis. *Cancer Cell* 2010;17:53-64.

- Menetrier-Caux C, Thomachot MC, Alberti L et al. IL-4 Prevents the Blockade of Dendritic Cell Differentiation Induced by Tumor Cells. *Cancer Res* 2001;61:3096-3104.
- Meyn RE, Stephens LC, Milas L et al. Programmed cell death and radioresistance. *Cancer Metastasis Rev* 1996;15:119-31.
- Milas L. Cyclooxygenase-2 (COX-2) enzyme inhibitors and radiotherapy: preclinical basis. *Am J Clin Oncol* 2003;26:66-9.
- Mirza A, McGuirk M, Hockenberry TN et al. Human survivin is negatively regulated by wild-type p53 and participates in p53-dependent apoptotic pathway. *Oncogene* 2002;21:2613-22.
- Miyata Y, Kanda S, Obah K et al. Lymphangiogenesis and angiogenesis in bladder cancer: prognostic implications and regulation by vascular endothelial growth factors-A, -C, and -D. *Clin Cancer Res* 2006;12:800-6.
- Murri A, Hilmy M, Bell J et al. The relationship between the systemic inflammatory response, tumour proliferative activity, T-lymphocytic and macrophage infiltration, microvessel density and survival in patients with primary operable breast cancer. *Br J Cancer* 2008;99:1013-19.
- Nagtegaal ID, Marijnen CA, Kranenbarg EK et al. Local and distant recurrences in rectal cancer patients are predicted by the nonspecific immune response; specific immune response has only a systemic effect--a histopathological and immunohistochemical study. *BMC Cancer* 2001;1:7.
- Nagtegaal ID, Marijnen CA, Kranenbarg EK et al. Short-term preoperative radiotherapy interferes with the determination of pathological parameters in rectal cancer. *J Pathol* 2002;197:20-7.
- Nakamura T, Mitomi H, Kikuchi S. Evaluation of the usefulness of tumor budding on the prediction of metastasis to the lung and liver after curative excision of colorectal cancer. *Hepatogastroenterology* 2005;52:1432-35.
- National Care Programs for Colorectal Cancer. Sweden 2008.
- O'Byrne KJ, Dalglish AG, Browning MJ et al. The relationship between angiogenesis and the immune response in carcinogenesis and the progression of malignant disease. *Eur J Cancer* 2000;36:151-69.

- O'Connor JK, Avent J, Lee RJ et al. Cyclooxygenase-2 expression correlates with diminished survival in invasive breast cancer treated with mastectomy and radiotherapy. *Int J Radiat Oncol Biol Phys* 2004;58:1034-40.
- Ocvirk J, Brodowicz T, Wrba F et al. Cetuximab plus FOLFOX6 or FOLFIRI in metastatic colorectal cancer: CECOG trial. *World J Gastroenterol* 2010;16:3133-43.
- Olumi AF, Grossfeld GD, Hayward SW et al. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer Res* 1999;59:5002-11.
- Pachkoria K, Zhang H, Adell G et al. Significance of Cox-2 expression in rectal cancers with or without preoperative radiotherapy. *Int J Radiat Oncol Biol Phys* 2005;63:739-44.
- Padera TP, Kadambi A, di Tomaso E et al. Lymphatic metastasis in the absence of functional intratumor lymphatics. *Science* 2002;296:1883-86.
- Palmqvist R, Oberg A, Bergström C et al. Systematic heterogeneity and prognostic significance of cell proliferation in colorectal cancer. *Br J Cancer* 1998;77:917-25.
- Parr C, Jiang WG. Quantitative analysis of lymphangiogenic markers in human colorectal cancer. *Int J Oncol* 2003;23:533-39.
- Parrott JA, Nilsson E, Mosher R et al. Stromal-epithelial interactions in the progression of ovarian cancer: influence and source of tumor stromal cells. *Mol Cell Endocrinol* 2001;175:29-39.
- Pawlik TM, Keyomarsi K. Role of cell cycle in mediating sensitivity to radiotherapy. *Int J Radiat Oncol Biol Phys* 2004;59:928-42.
- Peng CY, Graves PR, Thoma RS et al. Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of cdc25C on serine-216. *Science* 1997;277:1501-05.
- Peltenburg L. Radiosensitivity of tumor cells. Oncogenes and apoptosis. *Q J Nucl Med* 2000;44:355-64.
- Petersen OW, Nielsen HL, Gudjonsson T et al. Epithelial to Mesenchymal Transition in Human Breast Cancer Can Provide a Nonmalignant Stroma. *Am J Pathol* 2003;162:391-402.
- Phillips RK, Hittinger R, Blesovsky L et al. Local recurrence following 'curative' surgery for large bowel cancer: I. The overall picture. *Br J Surg* 1984;71:12-6.

- Ponz de Leon M, Di Gregorio C. Pathology of colorectal cancer. *Dig Liver Dis* 2001;33:372-388.
- Ponz de Leon M, Rossi G, Gregorio C et al. Epidemiology of colorectal cancer: the 21-year experience of a specialised registry. *Intern Emerg Med* 2007;2:269-79.
- Purdie CA, O'Grady J, Piris J et al. p53 expression in colorectal tumors. *Am J Pathol* 1991;138:807-13.
- Påhlman L. Improved Survival with Preoperative Radiotherapy in Resectable Rectal Cancer. *N Engl J Med* 1997;336:980-87.
- Påhlman L. Surgical aspects. Colorectal cancer; Current status and future perspectives, first nordic symposium, Böhm Offset Denmark 1999.
- Quadrilatero J, Hoffman-Goetz L. Physical activity and colon cancer. A systematic review of potential mechanisms. *J Sports Med Phys Fitness* 2003;43:121-38.
- Ragnhammar P, Hafstrom L, Nygren P et al. A systematic overview of chemotherapy effects in colorectal cancer. *Acta Oncol* 2001;40:282-308.
- Raza S, Lang, Aggarwal BB et al. Necrosis and glioblastoma: a friend or a foe? A review and a hypothesis. *Neurosurgery* 2002;51:2-12.
- Rearden A. A new LIM protein containing an autoepitope homologous to "senescent cell antigen". *Biochem Biophys Res Commun* 1994;201:1124-31.
- Reddy B, Engle A, Katsifis S et al. Biochemical epidemiology of colon cancers: effects of types of dietary fiber on fecal mutagens, acids, and neural sterols in healthy subjects. *Cancer Res* 1989;49:4629-35.
- Reddy B, Hirose Y, Lubet R et al. Chemoprevention of colon cancer by specific cyclooxygenase-2 inhibitor, celecoxib, administered during different stages of carcinogenesis. *Cancer Res* 2000;60:293-97.
- Ricci MS, Zong WX. Chemotherapeutic approaches for targeting cell death pathways. *Oncologist* 2006;11:342-57.
- Riekkki R, Parikka M, Jukkola A et al. Increased expression of collagen types I and III in human skin as a consequence of radiotherapy. *Arch Dermatol Res* 2002;294:178-84.
- Roberts P. Diagnosis and staging of colorectal cancer. Colorectal cancer current status and future perspective. Böhm offset Denmark 1999;3:49-55.

- Rosa J, Canovas P, Islam A et al. Survivin Modulates Microtubule Dynamics and Nucleation throughout the Cell Cycle. *Mol. Biol. Cell* 2006;17:1483-93.
- Roy N, Deneraux QL, Takahashi R et al. The c-IAP and c-IAP-2 proteins are direct inhibitors of specific caspases. *EMBO J* 1997;16:6914-25.
- Ruifrok AC, Mason KA, Lozano G et al. Spatial and temporal patterns of expression of epidermal growth factor, transforming growth factor alpha and transforming growth factor beta 1-3 and their receptors in mouse jejunum after radiation treatment. *Radiat Res* 1997;147:1-12
- Ryan BM, O'Donovan N, Duffy MJ. Survivin: A new target for anti-cancer therapy. *Cancer Treat Rev* 2009;35:553-62.
- Rödel CJ, Haas J, Groth A et al. Spontaneous and radiation-induced apoptosis in colorectal carcinoma cells with different intrinsic radiosensitivities: survivin as a radioresistance factor. *Int J Radiat Oncol Biol Phys* 2003;55:1341-47.
- Rödel F, Hoffmann J, Distel L et al. Survivin as a radioresistance factor, and prognostic and therapeutic target for radiotherapy in rectal cancer. *Cancer Res* 2005;65:4881-87.
- Rödel F, Hoffmann J, Grauenbauer GG et al. High survivin expression is associated with reduced apoptosis in rectal cancer and may predict disease-free survival after preoperative radiochemotherapy and surgical resection. *Strahlenther Onkol* 2002;178:426-35.
- Rønnov-Jessen L, Petersen OW, Kotliansky VE et al. The origin of the myofibroblasts in breast cancer. Recapitulation of tumor environment in culture unravels diversity and implicates converted fibroblasts and recruited smooth muscle cells. *J Clin Invest* 1995;95:859-73.
- Saad RS, Kordunsky L, Liu YL et al. Lymphatic microvessel density as prognostic marker in colorectal cancer. *Mod Pathol* 2006;19:1317-23.
- Sah NK, Munshi A, Hobbs M et al. Effect of downregulation of survivin expression on radiosensitivity of human epidermoid carcinoma cells. *Int J Radiat Oncol Biol Phys* 2006;66:852-59.
- Sakao S, Taraseviciene-Stewart L, Lee JD et al. Initial apoptosis is followed by increased proliferation of apoptosis-resistant endothelial cells. *FASEB J* 2005;19:1178-80.

- Sarela AI, Scott N, Ramsdale J et al. Immunohistochemical detection of the anti-apoptosis protein, survivin, predicts survival after curative resection of stage II colorectal carcinomas. *Ann Surg Oncol* 2001;8:305-10.
- Shabo I, Olsson H, Sun XF et al. Expression of the macrophage antigen CD163 in rectal cancer cells is associated with early local recurrence and reduced survival time. *Int J Cancer* 2009;125:1826-31.
- Sharma H, Sen S, Lo Muzio L et al. Antisense-mediated downregulation of anti-apoptotic proteins induces apoptosis and sensitizes head and neck squamous cell carcinoma cells to chemotherapy. *Cancer Biol Ther* 2005;4:720-27.
- Shia J, Guillem JG, Moore HG et al. Patterns of morphologic alteration in residual rectal carcinoma following preoperative chemoradiation and their association with long-term outcome. *Am J of Surg Pathol* 2004;28:215-23.
- Shin S, Sung BJ, Cho YS et al. An anti-apoptotic protein human survivin is a direct inhibitor of caspase-3 and -7. *Biochemistry* 2001;40:1117-23.
- Simon HB. The immunology of exercise. A brief review. *JAMA* 1984;252:2735-38.
- Simpson JA, Al-Attar A, Watson NF et al. Intratumoral T cell infiltration, MHC class I and STAT1 as biomarkers of good prognosis in colorectal cancer. *Gut* 2010;59:926-33.
- Sinicrope FA, Gill S. Role of cyclooxygenase-2 in colorectal cancer. *Cancer metastasis Rev* 2004;23:63-75.
- Schmidt-Ullrich RK, Valerie KC, Chan W et al. Altered expression of epidermal growth factor receptor and estrogen receptor in MCF-7 cells after single and repeated radiation exposures. *Int J Radiat Oncol Biol Phys* 1994;29:813-19.
- Smith JC. Carcinoma of the rectum following irradiation of carcinoma of the cervix. *Proc R Soc Med* 1962;55:701-2.
- Smyth MJ, Crowe NY, Godfrey DY et al. NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma. *Int. Immunol.* 2001;13:459-63.
- Sobin L, Gospodarowicz MK, Wittekind C. *TNM Classification of Malignant Tumors*, 7th Edition, Wiley-Blackwell 2009.

- Sonveaux PA, Brouet, Havaux X et al. Irradiation-induced angiogenesis through the up-regulation of the nitric oxide pathway: implications for tumor radiotherapy. *Cancer Res* 2003;63:1012-19.
- Soslow RA, Dannenberg AJ, Rush D et al. Cox-2 is expressed in human pulmonary, colonic, and mammary tumors. *Cancer* 2000;89:2637-45.
- Steinauer KK, Gibbs I, Ning S et al. Radiation induces upregulation of cyclooxygenase-2 (COX-2) protein in PC-3 cells. *Int J Radiat Oncol Biol Phys* 2000;48:325-28.
- Sutandyo N. Nutritional carcinogenesis. *Acta Med Indones* 2010;42:36-42.
- Swinson DE, Jones JL, Richardson D et al. Tumour necrosis is an independent prognostic marker in non-small cell lung cancer: correlation with biological variables. *Lung Cancer* 2002;37:235-40.
- The National Board of Health and Welfare. *Cancer i siffror*. Sweden 2009.
- Tsujino T, Seshimo I, Yamamoto H et al. Stromal myofibroblasts predict disease recurrence for colorectal cancer. *Clin Cancer Res* 2007;13:2082-90.
- Tu SP, Jiang XH, Lin MC et al. Suppression of survivin expression inhibits in vivo tumorigenicity and angiogenesis in gastric cancer. *Cancer Res* 2003;63:7724-32.
- Tu Y, Li F, Goicoechea S et al. The LIM-only protein PINCH directly interacts with integrin-linked kinase and is recruited to integrin-rich site in spreading cells. *Mol Cell Biol* 1999;19:2425-34.
- Tu Y, Li F, Wu C. Nck-2 a novel Src homology 2/3-containing adaptor protein that interacts with the LIM-only protein PINCH and components of growth factor receptor kinase-signaling pathways. *Mol Biol Cell* 1998;9:3367-82.
- Ueno H, Jones AM, Wilkinson KH et al. Histological categorisation of fibrotic cancer stroma in advanced rectal cancer. *Gut* 2004;53:581-86.
- Wallace ME, Smyth MJ. The role of natural killer cells in tumor control—effectors and regulators of adaptive immunity. *Springer Semin Immunopathol* 2005;27:49-64.
- Wang-Rodriguez J, Dreilinger A, Alsharabi G et al. The signaling adapter protein PINCH is up-regulated in the stroma of common cancers, notably at invasive edges. *Cancer* 2002;95:1387-95.

- Vanselow B, Eble MJ, Rudat V et al. Oxygenation of advanced head and neck cancer: prognostic marker for the response to primary radiochemotherapy. *Otolaryngol Head Neck Surg* 2000;122:856-62.
- Washington M. Colorectal Carcinoma: Selected Issues in Pathologic Examination and Staging and Determination of Prognostic Factors. *Arch Pathol Lab Med.* 2008;132:1600-7.
- Webb D, Parsons T, Horwitz A. Adhesion assembly, disassembly and turnover in migrating cells-over and over and over again. *Nat Cell Biol* 2002;4:97-100.
- Vermaas MF, Ferenschild T, Nuyttens JJ et al. Preoperative radiotherapy improves outcome in recurrent rectal cancer. *Dis Colon Rectum* 2005;48:918-28.
- Willett WC. Diet, nutrition and avoidable cancer. *Environ Health Perspect* 103 Suppl 1995;8:165-70.
- Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. *Nature* 2002;408:307-10.
- Wu G, Chai J, Suber TL, et al. Structural basis of IAP recognition by Smac/DIABLO. *Nature* 2000;408:1008-12.
- Xie WL, Chipman JG, Robertson DL et al. Expression of mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc Natl Acad Sci U S A* 1991;88:2692-96.
- Yang Y, Zhu J, Gou H et al. Clinical significance of Cox-2, Survivin and Bcl-2 expression in hepatocellular carcinoma (HCC). *Med Oncol* 2010;1-8. [Epub ahead of print]
- Yonemura Y, Endo Y, Tabata K et al. Role of VEGF-C and VEGF-D in lymphangiogenesis in gastric cancer. *Int J Clin Oncol* 2005;10:318-27.
- Zhang H, Evertsson S, Sun XF et al. Clinicopathological and genetic characteristics of mucinous carcinomas in the colorectum. *Int J Oncol* 1999;14:1057-61.
- Zhang T, Otevrel T, Gao Z et al. Evidence that APC regulates survivin expression: A possible mechanism contributing to the stem cell origin of colon cancer. *Cancer Res* 2001;61:8664-67.
- Zhang Y, Chen K, Tu Y et al. Assembly of the PINCH-ILK-CH-ILKBP complex precedes and is essential for localisation of each component to the cell-matrix adhesion sites. *J cell Sci* 2002;115:4777-86.

Zhou M, Gu L, Li F et al. DNA damage induces a novel p53-survivin signaling pathway regulating cell cycle and apoptosis in acute lymphoblastic leukemia cells. *J Pharmacol Exp Ther* 2002;303:124-31.

Zlobec I, Lugli A. Prognostic and predictive factors in colorectal cancer. *Postgrad Med J* 2008;84: 403-11.