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MOLECULAR STUDIES OF IRRADIATION AND SN-38 ON COLORECTAL CANCER

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Vi upptäckte mer och mer
och jorden blev större och större.
Upptäckte ändå mer
och jorden blev bara en prick,
en liten leksaksballong
i oändligheten.

Större och mindre
av Nils Ferlin, 1957

ABSTRACT

Colorectal cancer (CRC) is one of most common cancer diseases worldwide. In Sweden approximately 5,000 new cases of CRC are generated each year, which makes it the third most common cancer disease among both men and women. The past decades of improved treatment strategies have considerably increased the five-year survival for CRC patients. However more could be achieved in this area, in particular for metastatic CRC, which is the cause of most CRC-related deaths. Therefore it is important to study the biological response to certain treatments induced in CRC to find valuable predictive and/or prognostic factors to select patients for better suited treatments.

The aim of this thesis was to gain insight into the molecular changes that occur following irradiation or treatment with SN-38 in rectal cancer patients or colon cancer cell lines by studying the RNA expression, protein expression, DNA cell cycle distribution and apoptotic response. The expression of phosphatase of regenerating liver (PRL) proteins was investigated in rectal cancers from 125 patients included in a randomized clinical trial of preoperative radiotherapy (RT). Increased expression of PRLs was seen at the invasive margin of primary and metastatic cancers compared with the inner area of the tumors. Moreover, strong PRL staining at the invasive margin correlated to distant recurrence and worse survival of patients in the RT group but not in non-RT group (Paper I). Radiosensitivity was studied by treating KM12C, KM12SM and KM12L4a colon cancer cell lines with radiation. KM12C is of low metastatic nature compared with the highly metastatic KM12SM and KM12L4a. Upregulation of ΔNp73 and PRL-3 might contribute to the radioresistant phenotype in KM12C. In contrast, KM12L4a shows a high frequency of apoptosis and lack of upregulation of ΔNp73, PRL-3 and survivin, which might explain its radiosensitive phenotype (Paper II). KM12C, KM12SM and KM12L4a were treated with SN-38 which inhibits topoisomerase 1 (topo-1). The results show that SN-38 induces G2/S arrest and possess the capacity to trigger apoptosis in the three cell lines (Paper III). To further elucidate SN-38 effect on these cell lines, the gene expression profile following SN-38 treatment was studied. Oligonucleotide arrays consisting of ~27,000 spots were hybridized with sample and reference cDNA. Both unsupervised and supervised hierarchical clustering analysis, and functional analysis were performed. Supervised hierarchical clustering gives a strong signal of 1453 discriminated genes, the vast majority being upregulated. Both upregulated and downregulated genes point toward a favorable impact of SN-38 regarding the apoptotic pathways. For example *RhoB* and *Bax* are upregulated together with downregulation of *Kras* and *survivin*, which promotes apoptosis (Paper IV).

In conclusion, PRLs may be valuable biomarkers for RT resistance, predicting a poor prognosis in rectal cancer patients. Targeting radio-resistance factors, such as ΔNp73 and survivin may contribute to an increased sensitivity to RT. SN-38 affects cell proliferation and apoptosis.

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Varje år insjuknar i Sverige ungefär 5500 personer i tjock- och ändtarmscancer, som på fackspråk benämns som kolorektalcancer. Det gör denna cancerform till den tredje vanligaste i Sverige hos både män och kvinnor, näst efter prostata- och bröstcancer. Tidiga symtom på sjukdomen är t.ex. ändrade tarmvanor samt slem och/eller blod i avföringen. Kosten och motionsvanor är exempel på faktorer som har betydelse för risken att insjukna i tjock- och ändtarmscancer, likväl som det genetiska arvet då ungefär 5-15 % av sjukdomsfallen är kopplade till en ärftlighet. Tjocktarmscancer behandlas med kirurgi i kombination med cellgifter och ändtarmscancer med strålning, kirurgi och cellgifter. Det är främst genom framsteg inom de olika behandlingsområdena som dödligheten för tjock- och ändtarmscancer har minskat under de senaste årtionden.

För att kunna förbättra både behandlingar och prognos av tjock- och ändtarmscancer är det viktigt att vi studerar biologiska mekanismer hos cancercellen. Med biologiska mekanismer menas hur cancercellen fungerar och genom att förstå det kan vi också förstå hur den är, eller kan bli, sårbar. Viktigt är också att ge varje patient rätt behandling. Eftersom olika tumörceller har olika biologiska profiler, är de inte sårbara på exakt samma sätt. Förhoppningsvis kan vår forskning öka möjligheten till att i framtiden ge en ”skräddarsydd” behandling till tjock- och ändtarmscancerpatienter.

I den här avhandlingen har två olika behandlingar studerats, strålbehandling och irinotekan. Resultaten från den första studies visar att uttrycket PRL-proteiner påverkar hur patienterna svarar på strålbehandling. Patienter med ett högt uttryck av PRL har en ökad risk för lokala återfall samt en sämre prognos. Uttrycket av PRL är starkare i den invasiva kanten av tumören, ett område där många av de avgörande förändringarna för tumörutvecklingen sker. Detta stärker således uppfattningen om att PRL-proteiner är involverade i mekanismer som rör tumörprogression och invasion. Men vi strålbehandlade även koloncancer celler (cellinjer) och studerade sedan bl.a celldöd (apoptos) och proteinuttryck. I korthet kan resultaten sammanfattas följande; celler som har ett lågt uttryck av proteinerna ΔNp73, survivin och PRL-3 är mest strålningskänsliga.

För att efterlikna den process som sker i tumören efter irinotekan behandling använde vi oss av en experimentell modell där koloncancercellinjer behandlades med irinotekans aktiva metabolit, SN-38. Irinotekan hämmar ett protein, topoisomeras-1 (topo-1), som är involverat replikation och transkription av DNA. Resultaten från den tredje studien visar att kolocancerceller svarar på SN-38 behandlingen med ökad apoptos och cellcykelstopp, samt nedreglering

av topo-1. Vidare sker en uppreglering av Bax, som är ett vikigt protein i den apoptotiska processen. För att ytterligare studera effekten av SN-38, studerades även cellernas genetiska profil efter behandling. Resultaten visar att många apoptos-relaterade gener, t.ex. *survivin, DOK6, RhoB* och *Bax*, blir uppreglerade på ett fördelaktigt sätt som främjer apoptos.

Genom att undersöka varför cancercellerna reagerar som de gör vid olika typer av stimuli kan gamla behandlingsmetoder förbättras och nya skapas. Detta kommer förhoppningsvis leda till att överlevnadsstatistiken förbättras ytterligare för tjock- och ändtarmspatienter.

ABBREVIATIONS

5-FU	5-fluorouracil
Apaf-1	apoptosis protease activating factor-1
APC	adenomatous polyposis coli
ATM	ataxia telangiectasia mutated
Bax	Bcl-2 associated X protein
Bak	Bcl-2 antagonist/killer
Bcl-2	B cell lymphoma-2
BH	Bcl-2 homologue
Caspases	cysteine-dependent aspartate-specific proteases
cDNA	complementary DNA
CIN	chromosomal instability
CK	cytokeratin
CRC	colorectal cancer
Da	dalton, unit for molecular mass
DAB	3,3-diaminobenzidine-tetrahydrochoride
DAPI	4',6-diamidino-2-phenylindole
DCC	deleted in colon cancer
DNA	deoxyribonucleic acid
ds	double stranded
ECL	enhanced chemiluminescence
ECM	extra cellular matrix
FBS	fetal bovine serum
Gy	gray, SI unit for absorbed dose
IAP	inhibitor of apoptosis protein
ICC	immunocytochemistry
IHC	immunohistochemistry
HR	homologous recombination
HRP	horse radish peroxide
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
MDM2	mouse double minute 2
MEM	minimal essential medium
MMP	matrix metallo proteinase
NHEJ	non-homologous end joining
NF$\varkappa$B	nuclear factor $\varkappa$B
NSAIDs	non-steroidal anti-inflammatory drugs
p	short arm of the chromosome ("petit")
PRL	phosphatase of regenerating liver
PTPs	protein tyrosine phosphatases
PVDF	Polyvinylidene fluoride
q	long arm of the chromosome
RNA	ribonucleic acid

RT	radiotherapy
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SMAD	Sma and Mad homologue
SN-38	7-ethyl-10-hydroxy-camptothecin
SEK	Swedish krona (currency)
ss	single stranded
TNF	tumor necrosis factor
TNM	tumor node metastasis
Topo-1	topoisomerase 1
WB	western blot
VEGF	vascular endothelial growth factor
WHO	World Health Organization

LIST OF PUBLICATIONS

This thesis is based on the followings papers, refereed to in the text by their Roman numerals (I-IV).

I. **Wallin ÅR**, Svanvik J, Adell G, Sun XF.
Expression of PRL proteins at invasive margin of rectal cancers in relation to preoperative radiotherapy.
International Journal of Radiation Oncology Biology Physics 2006; 65: 452-458

II. Pfeifer D, **Wallin Å**, Holmlund B, Sun XF
Protein expression following gamma-irradiation relevant to growth arrest and apoptosis in colon cancer cells with mutant p53.
Re-submitted

III. **Wallin Å**, Svanvik J, Holmlund B, Ferraud L, Sun XF
Anticancer effect of SN-38 on colon cancer cell lines with different metastatic potential.
Oncology Reports 2008; 19: 1493-1498

IV. **Wallin Å**, Francis P, Nilbert M, Svanvik J, Sun XF
The mRNA profile of colon cancer cell lines after treatment with SN-38.
Submitted

INTRODUCTION

General background

According to WHO, 7.6 million people died of some type of cancer disease in 2005 (World Health Organization, 2007). Cancer is a disease in which cellular growth is out of control. When the normal boundaries of a cell are disrupted and it suddenly starts to divide in an uncontrolled manner, a neoplastic growth is ultimately formed. This cell transformation, from normal to cancerous, often arises from accumulated mutations in proto-oncogenes, tumor suppressor genes and genes involved in DNA repair, all of which are normally responsible for the homeostasis of the cell (proliferation) (Vogelstein and Kinzler, 2004). Findings from the ancient cultures of Greece and Babylonia show us that man has known of cancer for very long time. The etymology of cancer is Greek, from the word *karkinos*, which means crab. This probably refers to the resemblance between the spread pattern of malignant tumors and the claws of a crab, but also to the shape of an untreated breast cancer, which looks as if the breast has been attacked by a crab's claw.

Colorectal cancer

Colorectal cancer (CRC) is one of the most common forms of cancer in the Western world and the second most frequent cause of cancer-related deaths. In 2002, approximately 1 million new cases of CRC were diagnosed worldwide, with a mortality rate of about 50% (Ferlay, 2002). In Sweden CRC is the third most common form of cancer among men and women, with more than 5,000 new cases every year. Only prostate and breast cancer have higher incidences, (Cancerfonden, 2008). Among the Swedish population there is a lifetime risk of about 5% of developing CRC. The risk is age-related and increases from the age of 50. The incidence of CRC in Sweden has increased during the past 20 years, although mortality is decreasing. The 5-year survival rate has improved about 18 % for colon cancer and 26% for rectal cancer from 1960 to 1995 (Birgisson, 2005). This remarkable improvement is due to earlier detection and better treatment strategies, e.g. improved surgical skills and pre-operative radiotherapy (RT) (Dahlberg, 1999; Colorectal Cancer Collaborative Group, 2001; Rougier and Mitry, 2003). The annual cost of treating CRC patients is about 500 million SEK in Sweden (Socialstyrelsen, 2007). This makes CRC not merely a tragic disease for those afflicted, but also a severe financial burden on society.

Colorectal cancer is often mentioned as a lifestyle-dependent disease with a high incidence in industrial countries and a low incidence in developing countries. Known lifestyle-related risk factors for CRC include diets that are rich in red meat, physical inactivity, obesity, smoking, and large consumption of

alcohol. Preventive factors are diets that are high in vegetables and fruits, physical activity and regular intake of non-steroidal anti-inflammatory drugs (NSAIDs) (Giovannucci and Willett, 1994; Boyle and Leon, 2002). However, other important factors influence the risk of developing CRC, including age, family history of CRC, a personal history of inflammatory bowel diseases or adenomatous polyps, and the genetic disposition. Hereditary forms of CRC counts for approximately 5-10% of all cases, and the two most common forms are hereditary nonpolyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) (Weitz, 2005).

Etiology

In 1990 Fearon and Vogelstein proposed a pathway which shows us how CRC is developed (Figure 1)(Fearon and Vogelstein, 1990). This pathway has later been modified (Kinzler and Vogelstein, 1996), but it clearly points out that CRC progression has to overcome certain checkpoints in order to develop from a normal cell through adenoma into a metastatic tumor. The mutations that arise during the pathway are results of chromosomal instability (CIN), fragility, and response to chromosomal losses or translocational gains, mainly found at 5q, 17p and 18q. It is therefore highly probable that these areas harbor tumor suppressor genes.

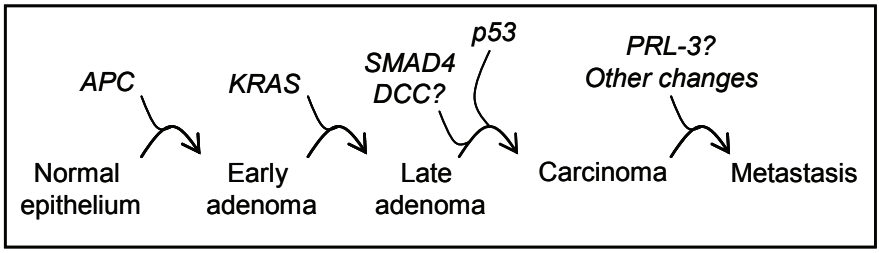

Figure 1. Genetic pathway for CRC development (adopted from Fearon and Vogelstein, 1990)

A carcinoma begins with an inactivation of the *adenomatous polyposis coli (APC)* gene located at 5q21, which encodes for a protein involved in cell proliferation. The *APC* is a gatekeeper gene, i.e. a tumor suppressor gene, and is frequently mutated (~60%) in sporadic cases of CRC. The inactivation leads to a transformation of the normal mucosa to an early adenoma. In general, this is followed by mutational inactivation of the oncogene (*KRAS)*, which causes the transition from an early adenoma into a late adenoma. The *KRAS* gene is located at 12p12 and is frequently mutated in other carcinomas. Although up to 60% of CRCs have a *KRAS* mutation, the clinical significance is still not clear. Significant for both mutations in the *APC* gene and in *KRAS* is that it only requires one mutational hit for inactivation, compared with *SMAD4* and *p53* inactivation. The tumor suppressor gene *p53* is inactivated in about 50% of all

colorectal tumors (Greenblatt, 1994). When this inactivation has taken place, the late adenoma can precede its transition into a carcinoma. Before that it also requires an additional inactivation step, for example of *SMAD4* and *DCC* genes, although these mutational inactivations are not as common as the other.

Stage

It is of clinical importance to stage each tumor, as it provides a valuable tool for prognosis and choice of treatment. All colorectal tumors are graded by a pathologist according to the Dukes or TNM stage (Table 1). The stage describes how deep the tumor has penetrated the wall of the intestine, whether or not it has spread to local lymph nodes and whether it has spread to distant organs. In 1932 the English pathologist, Dukes presented his staging system, known as Dukes stage (Dukes, 1932). This system has later been modified. It describes the growth development of the colorectal tumor, from Dukes A, when the tumor does not penetrate the muscularis propria, to Dukes D, when distant metastases are formed. Later, the classification called the TNM (tumor, node and metastasis) stage was introduced. The advantage of using the TNM stage compared with the older Dukes stage system is that the TNM considers the grades of tumor, nodes and metastases individually to give a more specific picture of the tumor status (Sobin and Fleming, 1997).

Table 1. TNM stage and Dukes stage in combination with 5-year survival

TNM stage		Dukes's stage	Description	5-year survival
0	TIS, N0, M0		Growth limited to the mucosa	> 95%
I	T1-2, N0, M0	A	Growth limited the wall of the intestine	85-100%
II	T3-T4, N0, M0	B	Penetration through muscularis propria.	50-75%
III	T1-4+any T, N1-2, M0	C	Regional lymph node metastasis	30-50%
IV	Any T, any N, M1	D	Distant metastasis to other organs	< 5%

Treatment

Surgery is the primary treatment for CRC. To reach complete remedy the tumor has to be surgically resected. The management, however, usually involves adjuvant chemotherapy. Preoperative radiotherapy is often used in rectal cancer patients and neo adjuvant chemotherapy is commonly used in advanced grades

of CRC. Downgrading may then allow curative resection. Palliative treatment includes primarily chemotherapy, although RT is used in some cases of rectal cancer (Regionalt Onkologiskt Centrum, 2002)

Radiotherapy

In Sweden, radiotherapy is given pre-operatively to rectal cancer patients in fractionated doses of 5 times 5 Gy, during a period of one week (Regionalt Onkologiskt Centrum, 2002). There are many advantages to using RT as adjuvant treatment. Primarily the tumor physically shrinks, facilitating the surgical resection. But the main advantage of pre-operative RT is the gain of local control. It has been shown that RT given preoperatively to rectal cancer patients decreases local recurrence by more than 60%, and as a consequence, overall survival is increased (Nagtegaal, 2005).

When the tumor is exposed to γ radiation, i.e. photons emitted in a de-excitation process, a chain reaction is started by photons hitting the tumor, resulting in the release of free radicals. Both single strand and double strand breaks (DSBs) in the DNA are formed due to the action of the free radicals, although DSBs are by far the most lethal (Leskov, 2001; Ricci and Zong, 2006). There are two different repair mechanisms for DNA DSBs in eukaryotic cells, homologous recombination (HR) and non-homologous end-joining (NHEJ). Both repair mechanisms are activated on DNA damage induced by ionizing radiation and act as complements to each other. Central to both repair mechanisms is the rapid activation of ataxia telangiectasia mutated (ATM) protein, which by phosphorylating several targets involved in cell cycle arrest, DNA repair and apoptosis, signals the presence of DNA damage. Downstream targets of ATM are for example p53, Mdm2 and Nbs1. Another key component of both HR and NHEJ is the Mre11/Rad50/Nbs1 complex which interacts with e.g. ATM (Khanna, 2001). In general, cells in the mitotic (M) phase are most sensitive to radiation followed by cells in G2, which are almost as sensitive (Figure 3) (Terasima and Tolmach, 1963; Sinclair and Morton, 1966).

SN-38

In the 1960s researchers revealed the cytotoxic truth of camptothecin. The toxic alkaloid, camptothecin, was extracted from the bark of the Chinese tree, *Camptotheca acuminta*, in the search for new cytotoxic agents (Wall, 1966). It was found to be effective in gastrointestinal cancers but was stopped in clinical trials in the 1970s due to its severe side effects, i.e. severe hematological cytotoxicity and diarrhea. The side effects are the results of camptothecin's water insolubility, and the substance was not re-used in clinical trials until irinotecan, a water-soluble analog was synthesized (Creemers, 1994). Irinotecan has been proved to have anti-tumor effects in a variety of tumor types including

colorectal, breast, lung, testicular and cervical tumors (Zamboni, 1998). Irinotecan is a prodrug that requires conversion by carboxylesterases to form the hydrolyzed active metabolite, 7-ethyl-10-hydroxy-camptothecin (SN-38) (Figure 2). Although irinotecan possesses some cytotoxicity itself, SN-38 is up to 1000-fold more active and therefore mainly responsible for the cytotoxic effect (Hertzberg, 1989; Hsiang, 1989). Irinotecan is used for chemotherapy in advanced CRCs that do not respond to or progrediate after fluorouracil (5-FU) treatment (Cunningham, 1998; Rougier, 1998).

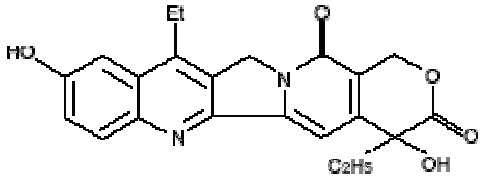

Figure 2. Chemical structure of SN-38

Camptothecin and its derivatives are all topoisomerase 1 (topo-1) inhibitors. Topo-1 is an enzyme involved in DNA replication and transcription. During the replication and transcription the super-coiled DNA helix needs to unwind. This is achieved through topo-1, which relaxes the strained helix by inducing temporary single-stranded DNA breakages that are later resealed by the enzyme (Alberts, 1994). In the presence of camptothecin the resealing is knocked out, subsequently leading to double stranded (ds) DNA breaks, permanent cell cycle arrest and finally cell death. The inhibitory effect derives from the complex formed between the drug molecules and the DNA strand (Hsiang, 1989). Due to the relationship between transcription and replication, the drug is most effective during the S-phase of the cell cycle (Figure 3).

Metastasis

As we learned from Fearon and Vogelstein, CRC progression ends with formation of distant metastasis. Metastasis is the spreading of malignant cancer cells to the surrounding tissue and subsequently to distant organs. This is a common feature of most cancers and the main reason for cancer-related deaths. In CRC, metastases are most commonly found in the liver, and approximately 50% of CRC patients develop liver metastasis. As early as 1889, Paget presented his "seed and soil" theory, in which he proposed that the formation of metastases is a non-random event, in contrast to the prevailing opinion. Certain tumor cells (the 'seeds') are predisposed to form new growths in certain organs due to the specific microenvironment (the 'soil') different organs provide. Although Paget's hypothesis is more than 100 years old, it is still

relevant, albeit in an updated form (reviwed in (Fidler, 2002). The hypothesis consists of three basic principles. First, primary neoplasms are heterogeneous in two different senses; they consist of both tumor cell and stromal elements, and they consist of genetic sub-clones. Second, cells that metastasize have to be successful in a series of steps, e.g. invasion, embolization, extravasation and, finally, survival and proliferation. Third, as Paget hypothesized, the 'soil' is decisive for the formation of metastasis as certain tumor cells can only metastasize in a specific microenvironment of an organ.

Certain key events lead to the formation of metastases. Tumor cells have to gain motility, they have to intravasate and extravasate, and they have to proliferate in the new environment (Sahai, 2007). The extracellular matrix (ECM) helps to retain the well-defined structure of the solid tumor and the loss of ECM associated proteins leads to disseminated tumor cells. Tumors often show overexpression of ECM-degrading proteases, e.g. matrix metalloproteinases (MMPs), promoting the step towards motility. Over expression of MMP-7 in CRC has been correlated to invasion and formation of liver metastasis (Zeng Z. S., 2002). Recruitment of lymphangiogenetic and angiogenetic factors not only provides the solid tumor with nutrients, but also facilitates new escape routes for motile cells. Once tumor cells have penetrated the walls of the lymphatic or blood vessels, the intravasation step is overcome and the tumor cells are free to circulate. Angiogenesis is the formation of new blood vessels, which in turn provides the solid tumor with both nutrients and oxygen. As vascular endothelial growth factor (VEGF) recruits and promotes proliferation of endothelial cells, it is a major factor contributing to angiogenesis (Hoshida, 2006). The intravasation step is followed by extravasation, which allows the cells to either proliferate in the new environment or to dormancy. However, most cells that arrive to a secondary site undergo apoptosis and non-metastatic cells are more prone to undergo apoptosis compared to their metastatic counterparts (Kim, 2004).

Cell cycle

The cell cycle is the life and death of a cell so far as it regulates cell proliferation, a mechanism that is often disturbed in cancer. The cell cycle consists of four different parts: the synthesis (S) phase in which the DNA is duplicated; M phase in which the cell mass is divided to form two new cells; and the two gaps (G1 and G2) that respectively precede the M and S phases. After cell division, the cell can either directly re-enter the cell cycle or proceed to G0 for temporary or final hibernation (Figure 3) (Alberts, 1994).

The cell cycle is a highly regulated process, controlled through its different checkpoints. Depending on the stimuli of these checkpoints, the cell is pushed forward through the cell cycle, or stopped for repair or execution of cell death.

The proliferation rate is also affected by either stimulation or inhibition of the checkpoints.

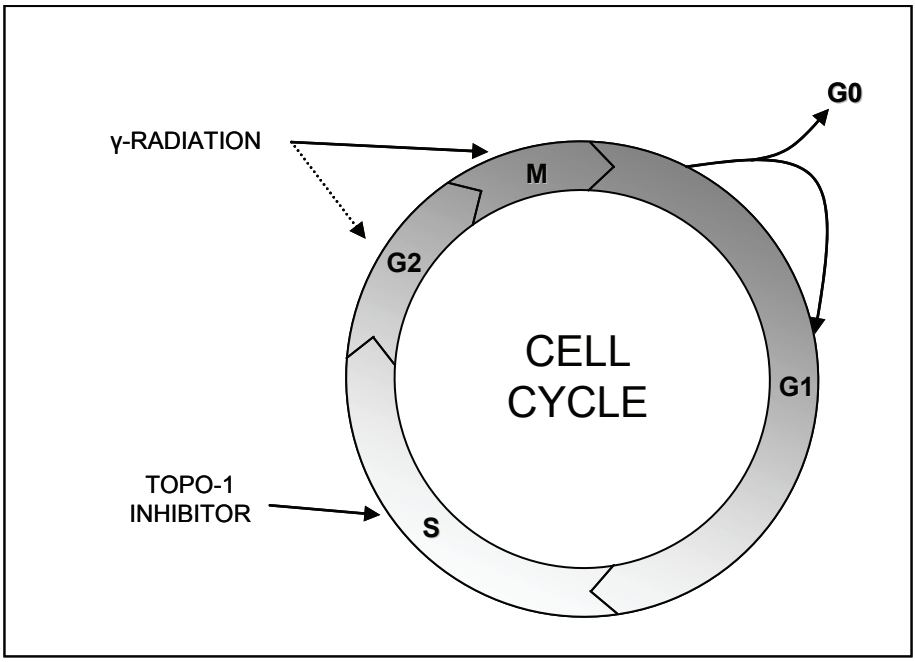

Figure 3. Stages of a cell cycle; starting with G1 and ending with the M phase. Radiation has mostly affects the M phase and topo-1 inhibitors cause changes at G2/S.

Apoptosis

In general cell death is divided into two different types, necrosis and apoptosis. The necrotic cell death originates from a severe cell injury, which causes the instant need to abort a cell. Although not energy-demanding, the process is unfavorable to surrounding tissue. When the cell swells, lyses and releases its cellular contents, the inflammatory response is switched on . In contrast, programmed cell death, apoptosis, is a well controlled pathway by the cell with minimal damage to the tissue and for this reason is also referred to as the sucidal program.of a cell. The etymology for apoptosis derives from the Greek apo, 'from', and ptosis, 'falling', and the phenomenon was first described in 1972 by Kerr et al. (Kerr, 1972). Typical morphological features of an apoptotic cell are cell shrinkage, membrane blebbing, chromatin condensation and fragmented DNA (Figure 4). The process ends with the formation of apoptotic bodies that are taken care of by the immune system, i.e. macrophages. The apoptotic system is not only used as a mechanism for damaged cells, but it is also of importance to the homeostasis of the tissue during embryogenesis.

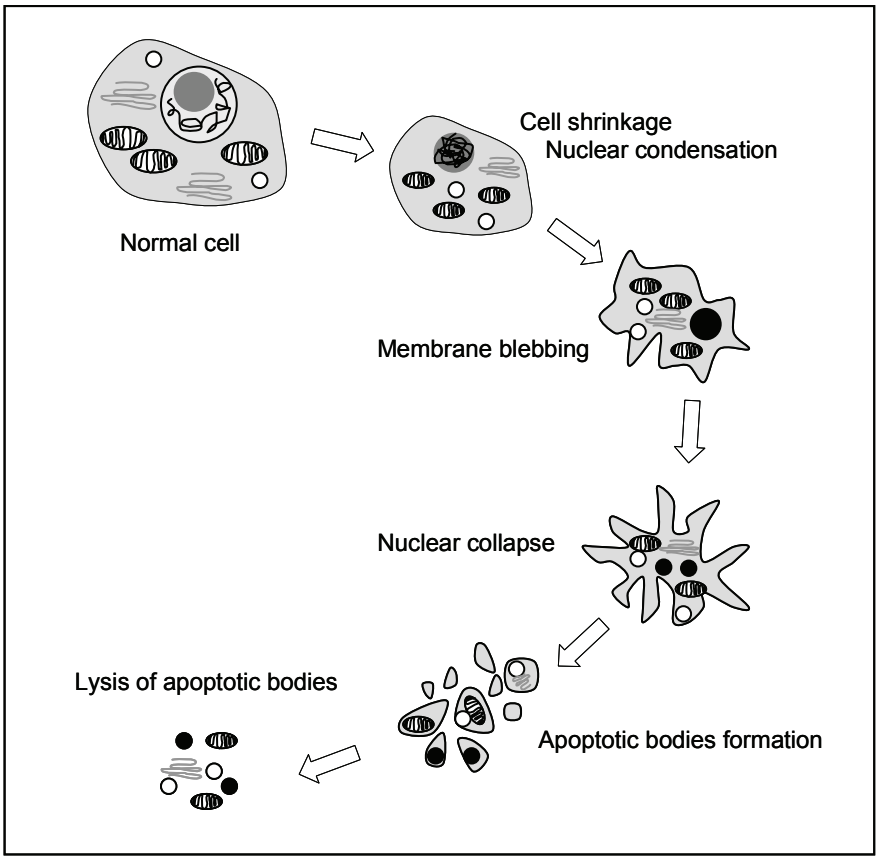

Figure 4. The apoptotic process; changes in the morphology

There are two different pathways of executing apoptosis as a response to cell death stimuli, and both of them result in a cascade activation of cysteine-dependent aspartate-specific proteases (caspases). Extrinsic signals trigger death receptors at the cell surface. The death receptors, Fas, tumor necrosis factor (TNF) receptor 1, and TRAIL, are included in the TNF super family and activated through ligand binding (Ashkenazi and Dixit, 1998). When activated, the mitochondrial pathway responds to intrinsic stimuli such as DNA damage, ischemia and oxidative stress. Upon stimulation, the mitochondrial outer membrane is permeabilized, resulting in pore formation. This disruption leads to a switch in the proton gradient, but it also allows water to flow inside the mitochondria, preparing for cytochrome *c* (cyt *c*) release. Together with apoptosis protease activating factor-1 (Apaf-1) and procaspase-9, cyt *c* forms an apoptosome, which in turn activates caspase 9 (Li P., 1997; Zou, 1999). The chain reaction continues with cleavage and activation of procaspases-3, -6 and -7. Caspase-3 is the link between the extrinsic and the intrinsic pathway, and

activation of procaspase-3 leading to conversion into caspase-3 is inhibited by inhibiting apoptosis proteins (IAPs) (Kaufmann and Hengartner, 2001).

IAPs regulate apoptosis. As their expression is often impaired in cancer, promoting cell proliferation, IAPs are potentially targets for chemotherapy (Wang, 2004). This is applied to enhance the existing capacity of RT and chemotherapy, but also for its direct cytotoxic effect. Survivin is a small anti-apoptotic protein of 16.5 kDa belonging to the IAP family (Ambrosini, 1997). Survivin is encoded by *BIRC*, located at the telomeric region of choromosome 17, and alternative splicing variants of the gene results in at least four different isoforms of survivin, of which three are full-length proteins (Mahotka, 1999; Badran, 2004). Wild type p53 can transcriptionally repress survin (Xia and Altieri, 2006) and the anti-apoptotic effects of survivin primarily derive from inhibition of caspase-3 and -7 (Pennati, 2007). Significantly, survivin is only expressed during embryonic development in normal tissue, with undetectable levels in normal adult tissue. Although it is expressed in many types of cancers, e.g. CRC, survivin constitutes an excellent target for therapeutics and is implicated in CT and RT response (Zaffaroni, 2005). Several knockdown studies show that downregulation of survivin promotes apoptosis and inhibits cell growth (Cheng, 2005; Miao, 2007; Zhen, 2007). The role as a predictive marker is still, however, unclear, but there is a prognostic value of survivin. Survivin is also widely implicated in the radiation response and suggested as a radioresistance factor (Capalbo, 2007). Rectal cancer patients with upregulated expression of survivin, displays an inverse relation of spontaneous apoptois and have an increased risk of local tumor recurrence after γ-radiation (Rodel, 2002). In colon cancer cells after γ-radiation, downregulation of survivin results in increased levels of G2/M and of DNA DSBs, indicating a favorable relation to apoptosis (Rodel, 2005).

The B-cell lymphoma-2 (Bcl-2) family consists of both pro-apoptotic members such as Bax and Bak, and anti-apoptotic members, such as Bcl-2 and Bcl-xl. Significant for the family members are the shared Bcl-2 homology domains (BH1-BH4) (reviewed in (Kirkin, 2004). The Bcl-2 protein family is subdivided into three groups, based on their apoptotic function and the domains they possess. Bax was the first pro-apoptotic member of the Bcl-2 family to be identified (Oltvai, 1993) and is located on chromosome 19q13, encoding a protein of 21 kDa (Apte, 1995). On stress activation, Bax forms a homodimer that induces cyt *c* release from the mitochondria (Gross, 1998; Antonsson, 2000; Antonsson, 2001). Upregulation of Bax can be induced via p53 (Miyashita and Reed, 1995) and Bcl-2 inhibits Bax activation (Oltvai, 1993). In CRC, an increase of Bax expression from normal to primary tumor has been detected (Krajewska, 1996; Miskad, 2004), as has decreased expression from primary tumor to metastases (Jansson and Sun, 2002). Bax is also of prognostic value in CRC, where low expression is correlated to a worse prognosis (Schelwies, 2002). Due to the character of a key component in the

mitochondrial pathway of apoptosis and the frequently altered Bax expression in cancer, Bax constitutes an excellent target for sensitization to both chemotherapy and RT. Supporting evidence indicates that Bax overexpression leads to enhanced drug sensitivity and increased apoptotic response (Guo B., 2000). Bax is also suggested as a predictive marker for RT in rectal cancer (Chang, 2005).

The p53 family

Activation of tumor suppressor p53 is essential for maintaining the integrity of the genome and in approximately 50% of all CRC cases, p53 mutations can be identified (Greenblatt, 1994). The p53 protein is involved in several cellular processes like DNA repair, cell cycle arrest and apoptosis. In normal cells p53 is expressed at low levels. Cellular stress, induced by for example ionizing radiation or DNA damaging agents, leads to activation of p53. Mdm2 is one important negative regulator of p53. However, Mdm2 is activated by p53 which leads to a negative feed-back loop with p53 (Vousden and Lane, 2007).

The p53 family also consists of the structurally like members p63 and p73. The DNA binding domains of the p53 family members shares a high sequence identity, which enables p63 and p73 to transactivate p53-responsive genes. Studies of knockout mice show that the biological functions vary between *p53, p63* and *p73*. Not only have p63 and p73 similarities of p53's tumor suppressor activity, they are also essential in development and differentiation control (Stiewe, 2007; Tomasini, 2008).

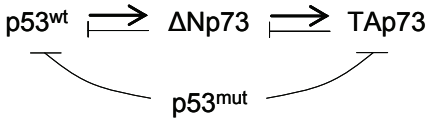

Figure 5. The auto-regulatory loop of p73. TAp73 is controlled by ΔNp73, which in turn is induced by both $p53^{wt}$ and TAp73, repressing their actions. Certain mutant forms of p53 binds to TAp73 and inhibits its actions. The expression of $p53^{wt}$ is regulated by ΔNp73 and the Mdm2 negative feed-back loop.

In contrast to p53, p73 is rarely mutated in tumors and p73 can activate promoters of several p53-responsive genes like p21, Bax, Mdm2 and GADD45. Therefore p73 has been suggested to have an important role in the restoration of p53, when function of p53 is impaired (Zhu, 1998; Davis and Dowdy, 2001; Liu S. S., 2004). The gene *p73* harbors two different promoter regions, which results in two different isoforms, TAp73 and ΔNp73. Both are full length, transcriptionally competent proteins, however ΔNp73 is N-terminally truncated and consequently lacks the transactivating domain (Coates, 2006). Instead of a p53 function, ΔNp73 is attributed a regulatory function (Figure 5). ΔNp73

downregulates both Tap73 (by protein-protein interaction) and p53 (by DNA competitive bindning), and is downregulated by the same genes, creating an autoregulatory loop. Moreover, certain mutated forms of p53 can bind to and inhibit expression of $p53^{wt}$ and Tap73 (Gaiddon, 2001; Bensaad, 2003). In various tumors, ΔNp73 are the most abundant form of the p73 proteins and silences the pro-apoptotic function of Tap73 (Concin, 2004). Overexpression of ΔNp73 has been related to a more radioresistant phenotype in cervical cancer (Liu S. S., 2004; Liu S. S., 2006).

PRL-3

In general, protein tyrosine phosphatases (PTPs) play a fundamental role in regulating diverse cellular processes, e.g. growth, differentiation and transformation, and the phosphatase family of regenerating liver (PRL) proteins is no exception. The PRL protein family comprises three members, PRL-1, PRL-2 and PRL-3 (also known as PTP4A1, PTP4A2, PTP4A3). The genes encoding these proteins are located on chromosomes 6q12 (*PRL-1*), 1p35 (*PRL-2*) and 8q24.3 (*PRL-3*). PRL-1 was the first to be identified and gets its name from its increased expression in regenerating liver. The amino acid sequence identity is high between PRLs in humans. PRL-1 and PRL-2 share 86% of their identity, PRL-1 and PRL-3 78%, and PRL-2 and PRL-3 at least 75%. They also share a COOH-terminal prenylation motif (reviewed in (Stephens, 2005; Bessette, 2008). Normally, PRL-3 expression is predominantly found in the muscle and heart of human adult tissue. In contrast, PRL-1 and PRL-2 are ubiquitous expressed in human tissues (Dumaual, 2006).

The relationship between cancer and PRL protein has been shown in different ways. As regeneration is a process of a highly proliferative state, this was the first indication that impaired PRL-1 expression may influence tumorigenesis. It is also shown that overexpression of PRL-1 and PRL-2 can transform mouse fibroblasts and pancreatic cells *in vitro* and promote tumor growth in nude mice (Diamond, 1994; Cates, 1996). The evidence for PRL-3 being involved in CRC metastasis was first discovered by Saha et al. (Saha, 2001). By performing a gene expression profile based on the SAGE library it was found that PRL-3 expression was markedly elevated in cells from liver metastases compared with non-metastatic colorectal tumors or normal colon epithelium. Since then, several studies have reported results that support this finding. Gene expression analysis showed mRNA overexpression of PRL-3 in metastatic colorectal and gastric cancers compared with non-metastatic colorectal and gastric cancers, which do not show this pattern (Bardelli, 2003; Kato, 2004). Further examination of protein expression has shown upregulation of PRL-3 in metastasis lesions of CRC compared with primary tumor or normal colorectal epithelia (Peng, 2004). *In vitro* studies in melanoma, ovary and gastric cancers, complement these findings, showing that the inhibition of PRL-3 by siRNA leads to the suppression of tumor progression whereas upregulation of PRL-3

facilitates metastasis (Kato, 2004; Polato, 2005; Qian, 2007). Angiogenesis is an important step promoting metastasis. By attracting endothelial cells that initiate angiogenesis, PRL-3 is also implicated in this critical step in the metastatic process (Parker, 2004). Strong evidences of PRL-3 involvement in cancer progression leads to the next question concerning PRL-3 as a potential target for chemotherapy. Recently Daouti et al. reported that a small molecule, 7-amino-2-phenyl-5H-thieno[3,2-c]pyridin-4-one (thienopyridone), selectively inhibits PRL-3, which leads to suppressed three-dimensional tumor growth *in vitro* (Daouti, 2008).

AIMS

The general aims of this thesis were to investigate how treatment affects the molecular biology of CRC cells and to identify available biological markers for therapy.

Paper I

To relate PRL expression and pre-operative RT in rectal cancer patients based on the protein expression and clinicopathological factors.

Paper II

To show the protein expression profile in colon cancer cells *in vitro* after irradiation and to evaluate potential effects in the p73 pathway.

Paper III

To demonstrate the effect of SN-38 treatment *in vitro* in colon cancer cells, with focus on the apoptotic response.

Paper IV

To view the genetic profile of colon cancer cells treated *in vitro* with SN-38.

MATERIALS AND METHODS

The following is a brief description of the materials and methods. For detailed information, please see Papers I-IV.

Ethical aspects

When working with research that includes biomaterial from human, there is always an ethical aspect. The Nuremberg Code states that the voluntary consent of the patient who is to participate in a trial is needed, but also that the research should be of benefit to society in general and that the risks for the participants must be minimized. It also states that participants are free to discontinue trials if they so wish and that the researcher responsible has to interrupt the trial if it is suspected that the participants may be endangered (Markman and Markman, 2007). The Nuremberg Code is the fundamental guideline for ethical committees in Sweden. In line with this, the patients included in this thesis have given consent for the material taken to be used in scientific research and the use of the material was approved by the Human and Ethical Committees at the Faculty of Health Sciences, Linköping, Sweden.

Patient material (Paper I)

The patients in this study participated in the Swedish rectal cancer trial of preoperative RT between 1987 and 1990. The material included 125 rectal cancer patients all of whom had primary cancer. Eighty-two of the specimens had adjacent normal mucosa, 36 had distant normal mucosa, of which 33 were matched cases to primary tumor (i.e. distant normal mucosa and primary tumor from the same patient), 30 had regional lymph nodes metastases, of which 28 cases were matched to primary tumor, and 96, with 74 matched primary tumor cases, had biopsy specimens from the primary tumor. Distant normal specimens were taken from the margin of the resection and were histologically free from both pre-tumor and tumor. The endoscopic biopsies were taken by the surgeons and constituted the basis for clinical diagnosis. If the biopsy was not qualified for the clinical diagnosis, another biopsy was taken. Of the 125 patients, 67 underwent tumor resection alone and 58 received pre-operative RT followed by tumor resection. The RT delivered with 6-10 MV photons was given as fractionated dose 5x5 Gy, within a median of 6 days and a range of 1-13 days.

Cell lines (Papers II-IV)

The cell lines used in this thesis were originally established in I.J. Fidler's laboratory (described in Figure 6) and kindly given to us.

To establish a metastatic colon cancer cell line, cells were collected from a colon cancer tumor of Dukes B to establish a primary colon cell line. From this cell line, cells were repeatedly injected into nude mice to get metastatic derivatives. Cells were injected directly into the spleen and to the cecum to mimic the spontaneous route metastatic cells take from the original site in the colon to the distant metastasis. The injected cells homed to the liver and formed liver metastases. The tumor growths formed in the liver were removed, disseminated, plated and established as two new metastatic cell lines, KM12SM and KM12L4a. KM12SM is the derivative in which cells were injected to the spleen and is said to posses a lower metastatic potential than its sibling KM12L4a, which originated from the cells injected into the cecum and therefore has with highest metastatic potential. Finally, the cell lines were examined to secure that they exclusively contain human cells (Morikawa, 1988).

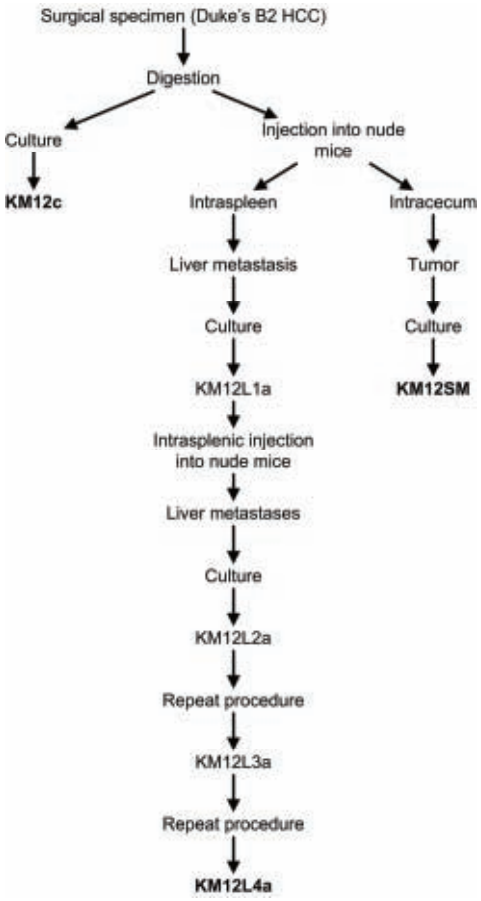

Figure 6. Schematic picture of the procedures used to obtain cell lines with different metastatic potential, KM12C, KM12L4a and KM12SM (adopted from Morikawa, 1998)

KM12 cell lines were cultured in Eagle's Minimal Essential Medium (MEM) with Earle's salts, L-glutamine and non-essential amino acids (Sigma-Aldrich, Stockholm, Sweden), supplemented with 1.5% $NaHCO_3$, 1mM Na-Pyruvate (Invitrogen, Carlsbad, CA), 1X MEM vitamin solution (Invitrogen), 5% Penicillin-Streptomycin (Invitrogen) and 10% fetal bovine serum (FBS; Invitrogen).

Treatments for *in vitro* studies

Radiation (Paper II)

The cell cultures were radiated with a single-dose of 10 or 15 Gy, doses that were chosen after the results of a pilot study. The criterion was to induce apoptosis that was adequate for testing the hypothesis, while remaining of biological interest. Cells were seeded at a density of 20,000 cells/cm^2 for all experiments, 24 h before they were irradiated with photons from a 4MV or 6MV linear accelerator Varian Clinac 600C or Varian Clinac 600C/D. The radiation area was 40x40 cm, and the distance between source and cells was 90 cm. Acrylic glass plates were placed both above (3 cm thick) and underneath (10 cm thick) the cells. A negative control (0 Gy) was included for all experiments and the cells were seeded in minimum duplicates. Finally, all experiments were repeated three times to produce stable results over time.

SN-38 (Papers III-IV)

Cells were treated with SN-38, a topo-1 inhibitor. A pilot study was conducted to test different concentrations and incubation times for the drug. The final concentration was 2.5 ug/ul (medium as diluent) and cell cultures were incubated with SN-38 for 4, 24 and 48 h, directly followed by harvesting. Untreated controls were used as reference sample for all experiments.

Immunohistochemistry and immunocytochemistry

For the visualization of protein localization and expression, the use of antibodies is fundamental (Figure 7). Immunohistochemistry (IHC) enables proteins in tissue (in situ) to be localized by using a specific antibody against the antigen (protein) of interest. The same method is referred to as immunocytochemistry (ICC) if the material consists of intact cells from a cell culture. For the present thesis we used a two-step immunohistochemical staining method. The tissue section is first incubated with a primary antibody that binds to the specific epitope of interest and then incubated with a secondary enzyme labeled antibody. The most common forms of enzyme labeling are horse radish peroxide (HRP) and alkaline phosphatase. In Paper 1 the DakoCytomation EnVisionTMSystem-HRP was used, which is a detection system that enhances the detection signals. In Paper III bionitylated secondary

antibody was used, which forms a complex with avidin-peroxidase. This complex is visualized by 3,3-diaminobenzidine tetrahydrochoride (DAB). Finally a counterstaining is performed and the slides are examined by light microscope. Stained slides were examined by two independent researchers, one of whom was a pathologist (Paper I). In cases of disagreement, the slides were re-examined until consensus was reached.

Western blot

Western blot is a protein detection method in which proteins are separated only by their molecular weight. The name derives from western, in contrast to the Southern and northern blots used to separate DNA and RNA, and to blot, as the proteins are transferred to a membrane via electrical tension. First, a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) separation is conducted, followed by a blotting step to transfer proteins to a nitrocellulose membrane (PVDF membrane). The membrane is incubated with a primary antibody and a secondary antibody conjugated to HRP (Figure 7). The result is detected by using a chemiluminescence method in which a light-sensitive film is exposed to the light reaction that is formed when HRP reacts with hydrogen peroxide and luminal in the enhanced chemiluminescence (ECL) system.

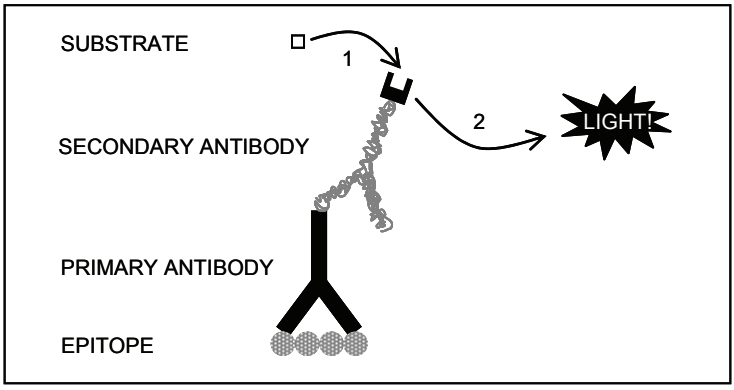

Figure 7. The basic principle for detection using antibodies.

Apoptosis analysis

There are many ways to detect apoptosis. One of the most basic methods is to simply examine the morphology of a cell by microscope. To enhance the visualization, the cells could first be stained with 4',6-diamidino-2-phenylindole (DAPI), a fluorescent molecule that binds to ds DNA and stains both metabolic and non-metabolic active cells. DAPI stains the DNA, making the morphology of the cell easier to analyze, i.e. the status of the nucleus. DAPI stained slides are examined by a fluorescence microscope.

The apoptosis was also examined by immunostaining with apoptotic marker M30. This application was used for both ICC and flow cytometry. Caspase-3, -6,-7 and -9 activity is detected by M30 monoclonal antibody. M30 is an antibody that binds to a neo-epitope of Asp396 after cleavage of cytokeratin (CK) 18 by caspase (Leers, 1999).

DNA flow cytometry

To analyze the cell cycle distribution DNA flow cytometry was performed. A modification of the Vindelöv protocol was used for this analysis. Basically, cells are stained with propidium iodine, an intercalating agent which is fluorescent, and then detected by flow cytometry (Vindelov, 1983).

Statistical analysis

To test the significance of the differences in PRL expression between the different tissues and the association of PRL expression with clinicopathologic/biologic variables, McNemar's or the chi-square method was used (Paper I). The relationship between PRL expression and survival was tested using Cox's proportional hazard model, including both univariate and multivariate analyses. The test was two-sided and $p< 5\%$ was considered statistically significant.

Pooled t-test was used in Paper III to test the deviation in apoptosis between treated and untreated cell lines, examining any time-dependency for the drug response and evaluating differences between the cell lines. The pooled t-test is used when the groups consist of a small number of cases (in Paper III, $n=2$). All p values below 5% were considered significant.

Gene expression analysis (Paper IV)

Microarray is advantageous in the examination of the gene expression profile as the expression of thousands of genes can be studied at the same time. There are several applications for microarray, and the expression profile can be analyzed at different levels, RNA, DNA and protein. In Paper IV the RNA expression is studied using cDNA microarrays that consisted of ~27,000 spots, each representing a sequence-verified IMAGE clone from the Research Genetics IMAGE clone library.

From the samples (treated cells and untreated controls), the RNA was isolated. Samples and reference RNA (the Universal Human Reference RNA, Stratagene, La Jolla, CA) were transcribed into cDNA, labeled with Cy3 and Cy5, respectively, and pooled together, along with blocking reagents. Hybridization was performed manually, and the slides were washed and sealed at 42°C for 18 hrs. The cDNA microarrays consisted of 27,648 spots, each

representing a sequence-verified IMAGE clone from the Research Genetics IMAGE clone library. The slides were manufactured at the Swegene DNA Microarray Resource Center, Department of Oncology, Lund University, Sweden. All slides were scanned, and the images were processed to correct for incorrect spots etc. All the image files were imported into the Web-based BioArray Software Environment (BASE; http://base.onk.lu.se/int/; ((Saal, 2002), in which all data management and analysis were executed. Background corrections, filtering, transformations and analyses were performed uniformly on the data. Unsupervised hierarchical clustering based on Pearson correlation distance was done in the software TMeV (www.tm4.org/mev.html; ((Saeed, 2003), and a supervised analysis based on the Golub score was also performed. To enable identification of functional groups, Gene Ontology categories and/or pathways EASE software was used (Hosack, 2003). Cut-off values of p= .001.

RESULTS AND DISCUSSIONS

Paper I

In this study the relationship between PRL protein expression and pre-operative RT in rectal cancer patients was investigated. The material consisted of 125 rectal cancer patients, randomized in a clinical trial to receive either pre-operative RT followed by surgical resection or surgery alone. Examination of the expression was carried out by immunohistochemical staining of distant normal mucosa, adjacent normal mucosa, primary tumor, biopsy specimens and lymph node metastasis. The antibody used for this study was provided by Prof. Bert Vogelstein, and although it was defined as PRL-3 specific, a specificity for all the three family members, PRL-1, PRL-2 and PRL-3, was later discovered. However, as all three PRL proteins are implicated in cancer, due to their involvement in cell proliferation and to some extent also to metastasis, the expression is further discussed as expression of PRL, referring to PRL-1, PRL-2, PRL-3, in this paper.

The PRL expression was located in the cytoplasm for both normal epithelia and tumor cells. Nuclear staining was absent in the normal epithelial cells and found in only two tumor cases (one primary tumor and one lymph node metastasis), which displayed both nuclear and cytoplasmic staining. The character of the staining was heterogeneous and granular, especially for the tumor cells. No stromal staining was detected.

The frequency of PRL expression increased from distant normal mucosa to adjacent normal mucosa ($p<.0001$), and from distant normal mucosa to primary tumor ($p=.002$). There was no difference in expression between primary tumor and metastasis, although adjacent normal mucosa had a higher frequency of strong PRL expression compared with primary tumor ($p<.0001$). When comparing the matched cases, i.e. the samples of normal mucosa, primary tumors and metastases from the same patient, the same pattern of increased PRL expression in adjacent normal mucosa and primary tumor compared with distant normal mucosa was displayed. And again no difference in expression was found between primary tumor and metastasis. The PRL expression at the invasive margin was also analyzed in relation to clinicopathological factors. In the RT group strong margin expression was related to poor survival ($p=.01$) and to distant recurrence ($p=.006$). No such relationships were found in the non-RT group (Figure 8). However, relationships of Ki67 expression ($p=.001$) and necrosis ($p=.049$) were found, indicating that PRL proteins were associated with a highly cell proliferative state. PRL proteins are believed to play a key role in the regulation of cellular growth and the cell cycle (Neel and Tonks, 1997; Hunter, 2000; Zeng Q., 2000).

Multivariate analysis showed only correlation with prognostic significance of PRL expression in the RT group. A similar trend was found between strong margin expression in lymph node metastasis and decreased patient survival. Therefore, PRL proteins might play the role of a radioresistant factor.

The increase in PRL expression from distant normal mucosa to primary tumor together with the strong expression at the invasive margin of both primary and metastatic tumors supports the hypothesis of PRL as a biological factor involved in tumor development and invasiveness. Adjacent normal mucosa shows the highest PRL expression. The reason for this is unknown, although the adjacent normal mucosa is potentially an area of intermediate biological changes and increased risk of neoplastic growths (Lipkin, 1988).

At the invasive margin, morphological and biological changes occur that are of importance to tumor development and progression. Tumors with infiltrative growth pattern at the invasive margin display a more malignant phenotype, with a poorer prognosis for CRC patients, compared with tumors with an expansive growth pattern (Ono, 1996; Fujita, 2003). In this paper the expression of PRL at the invasive margin was compared with expression of the protein in the inner part of the tumor. It was found that 38% of the primary tumors and 26% of the metastases showed stronger staining at the invasive margin. Notably, no specimen had stronger PRL expression in the inner part of the tumor thanthe invasive margin had. PRL-3 expression has been found in blood vasculature, indicating a role for PRL-3 in angiogenesis and lymph angiogenesis (Guo K., 2004; Parker, 2004). The invasive margin, the 'frontier' in tumor progression toward metastasis, makes it a target for angiogenesis and lymphangiogenesis, processes required for invasion and the formation of metastasis. It has been proposed that PRL-3 acts as a signal to promote the growth of new blood vessels and is involved in the degrading of the basal layer of the blood vessels to enhance the escape of penetrating tumor cells into the bloodstream (Guo K., 2004). An association between PRL-3 expression and lymphatic invasion has also been demonstrated in gastric cancer (Miskad, 2004). Moreover, it has been shown that Chinese hamster ovary cells expressing PRL-3 are more motile and have a higher invasive activity, and that PRL-3 expressing cells in mice are more prone to form tumor metastasis (Zeng Q., 2003; Guo K., 2004). This coincides with our findings of strong PRL expression at the tumor invasive margin, which was correlated to a more malignant phenotype and independently related to poor prognosis. Consequently, we propose that the PRL protein has a frontier role at the invasive margin as a tumor growth accelerator and invasive inducer.

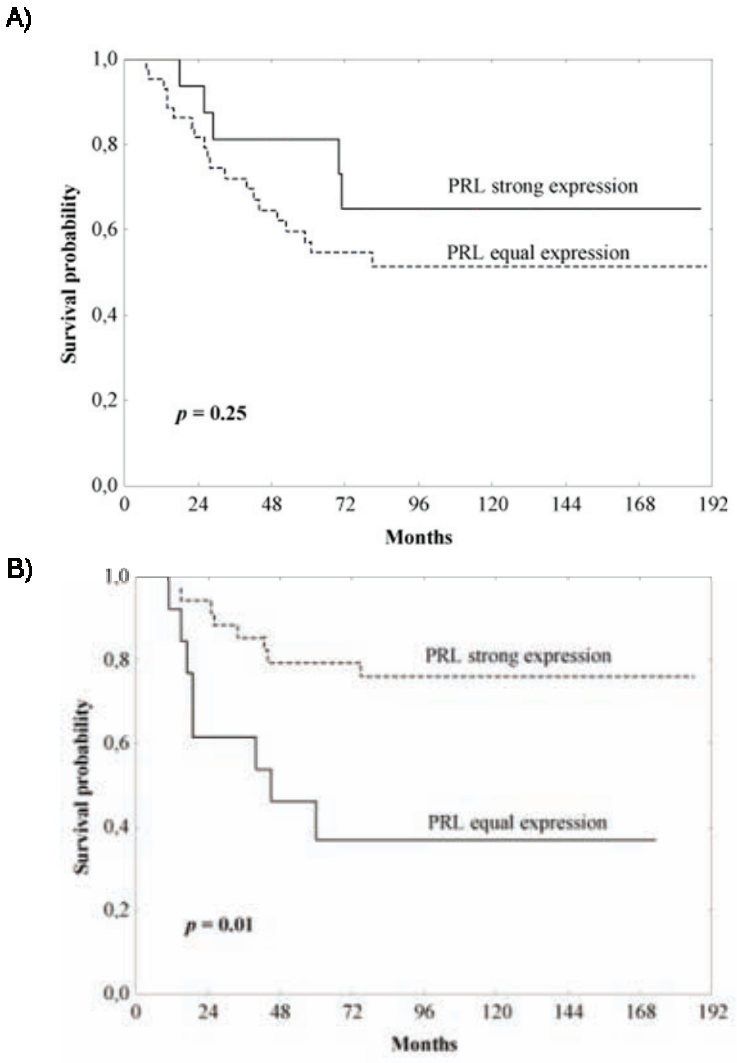

Figure 8. Phosphatase of regenerating liver (PRL) expression at tumor invasive margin in relation to survival in rectal cancer patients (A) without and (B) with preoperative radiotherapy (Wallin, 2008).

Paper II

In this study, colon cancer cell lines were radiated at single doses of 0, 5 and 15 Gy with γ-radiation. The expression of proteins related to cell proliferation and apoptosis was examined to further investigate the mechanism of the response of colon cancer cells to radiation (Table 2). Further, based on the results the intrinsic radiosensitivity of the three cell lines, KM12C, KM12SM and KM12L4a were clarified.

Table 2. Summary of the results from DNA flow cytometry, apoptosis analysis and protein expression analysis in Paper II.

	KM12C		KM12SM		KM12L4a	
	10Gy	15Gy	10Gy	15Gy	10Gy	15Gy
Cell cycle arrest	+	++	+++	+++	?	?
Apoptosis	+	+	+	+	++	++
TAp73	+	+	-	-	+/-	+
ΔNp73	+	+/-	-	+/-	-	-
P53 mut	+/-	+/-	+	+	+/-	+/-
Survivin	+/-	+/-	+	+	+/-	+/-
PRL-3	+	+	-	-	+/-	+/-

Radiation causes DNA damage that eventually effects proliferation and ultimately can lead to cell death (Dizdaroglu, 1992). Considering just the apoptotic response after irradiation, KM12L4a displays the highest rate of apoptosis. However, a colony-forming assay was performed as well to determine intrinsic radiosensitivity among the cell lines. The result showed a higher sensitivity to KM12C due to its enhanced ability to form colonies after radiation. Even though the results from the apoptotic and clonogenic analyses are adverse, they may not be controversial. KM12C has the shortest proliferation cycle, i.e. is the fastest growing cell line compared with KM12SM and KM12L4a. Previously, Nakayama et al. studied the colony-forming ability of the three cell lines and reported that KM12C only formed colonies to half the extent of the other two cell lines (Nakayama, 1998). In summary these two different methods of estimating the response to irradiation might not point in the same direction. They may, however, complement each other, pointing to different mechanisms. By studying the distribution of cells in the cell cycle, the effect on proliferation is investigated. The parental KM12C shows diploidy and response to irradiation, with a transient G2 arrest in contrast to the metastatic counterpart KM12SM, which displays near tetraploidy and permanently arrests in G2. KM12L4a consisted of two subpopulations, one diploid and one almost tetraploid. As a result, exact calculations of the cell distribution become

unfeasible due to the overlapping cell cycle phases. An effect observed on irradiated cells is a phenomenon referred to as 'giant cells'. This is a typical morphological feature of cells undergoing mitotic catastrophe, which is a result of a premature entry into mitosis (Mackey, 1988). The incomplete mitosis leads to an aberrant amount of DNA. In general the mitotic catastrophe cells are non-proliferative, although it has been demonstrated that smaller fractions can survive long enough to establish new growing colonies(Ricci, 2006). The abrogation of the G2/M checkpoint could, for example, derive from inactive p53 as mitotic catastrophe is mostly observed in cells lacking functional p53 (Ianzini, 2006). This strengthens the function of mitotic catastrophe as a mechanism for removing stress-induced damaged cells with impaired p53 control of the G2/M checkpoint. Both KM12C and KM12SM display giant cells after irradiation, and at 10 Gy KM12C displays fewer giant cells. Due to the absence of a morphological examination, it is not possible to determine whether the aberrant DNA content actually has the character of multinuclei, which is typical of mitotic catastrophe. However, because mitotic catastrophe is a common type of cell death after radiation, it is likely that cells seen as an additional peak of cells with a high DNA content in the cell cycle analysis curves are mitotic catastrophe cells. Thus the result indicates a tendency toward a more radioresistant phenotype for KM12C than for KM12SM.

The p73 gene harbors two promoter regions resulting in two different variants: TAp73, which is structurally like p53; and ΔNp73, which lacks the transactivation domain as well as the resemblance to p53 (Coates, 2006). In contrast to p53, p73 is rarely mutated in tumors and is therefore thought to compensate for lost p53 function (Liu S. S., 2004). The three proteins, p53 and the two isoforms of p73, are transcriptionally controlled by each other and are involved in an autoregulatory loop (Grob, 2001). ΔNp73 downregulates both p53 and TAp73 but is also upregulated by the same proteins. Moreover, TAp73 is inactivated not only by ΔNp73, but also by different mutated forms of p53 (Gaiddon, 2001; Bensaad, 2003; Li Y. and Prives, 2007). The results from Paper II display a relationship of decreased Np73 and an increase of pro-apoptotic TAp73. After irradiation the antiapoptotic Np73 is downregulated coincidently with a desired upregulation of TAp73. In cervical cancer, relationships between overexpression of Np73 and radioresistance have been shown, and increased expression of TAp73 and radiosensitivity (Liu S. S., 2004; Liu S. S., 2006). Consequently this indicates a p53 compensational function of TAp73 and that the p73 isoforms are potential biomarkers for RT. The IAP survivin blocks the apoptotic pathway and is normally not expressed in human adult tissue. However, survivin is frequently expressed in tumors, and there is substantial evidence for survivin being a radioresistant factor (Kami, 2005; Rodel, 2005; Sah, 2006). In Paper III the survivin expression was examined, and notably KM12SM shows a slight upregulation of survivin together with a decrease in apoptosis compared with the two other cell lines. The upregulation might be explained by the permanent G2 arrest because survivin is expressed mainly

during this phase of the cell cycle (Davis, 2001). Expression analysis of the metastasis-associated protein PRL-3 showed downregulation, or the absence of expression, for the two apoptosis sensitive cell lines, KM12SM and KM12L4a. In contrast, KM12C shows upregulation of PRL-3, which has been shown to be involved in cell cycle regulation due to its location on the metaphase plate, mainly affecting the passage through mitosis (Stephens, 2005). This might explain why KM12c is the only cell line showing upregulation of PRL-3 after irradiation as KM12C exclusively recovers and passes through the M phase. Also notable is the evidence for the upregulation of PRL-3 by TAp73 and p53. The KM12C cell lines carry a p53 mutation (Okamoto, 2000) and probably lack the ability to affect PRL-3 through p53. This study found that not only did KM12C show an upregulation of both TAp73 and PRL-3 but also that KM12SM showed downregulation of both TAp73 and PRL-3.

Paper III

To further investigate the molecular effect of irinotecan, colon cancer cells treated with SN-38 *in vitro* were analyzed for cell cycle distribution, apoptosis and apoptosis-associated proteins. Untreated controls were used as references. Although irinotecan possesses some cytotoxicity itself, it is a prodrug that requires conversion by carboxylesterases to form the active metabolite SN-38 (Hertzberg, 1989; Kawato, 1991). In this study, therefore, we used SN-38 treatment as a model for studying the indirect effect of irinotecan treatment of colon cancer cells.

SN-38 as a topo-1 inhibitor induces cell cycle arrest by stabilizing the topo-1 cleavable complexes formed during transcription. This leads to DNA damage followed by S phase arrest and subsequently G2 arrest (Hsiang, 1985). The cell lines used in this study showed different karyotypes. KM12C and KM12SM were diploid in contrast to KM12L4a, which showed tetraploidy. The cell cycle distribution of cells also differed between the three cell lines. KM12C and KM12SM displayed similar patterns of increased S phase arrest after SN-38 treatment for 24 and 48 h, and decreased G2 arrest after 4 and 24 h. The result for KM12L4a was similar with respect to S phase arrest, with increased fraction of cells in S phase for all three time points. However, G2 distribution differed from that of the other two cell lines. KM12L4a displayed a pattern of an increased number of cells in G2, this in a time-dependent manner, following SN-38 treatment, i.e. an accumulation of cells. This phenomenon may be explained by the tetraploidy KM12L4a displays. As the drug is a DNA-damaging agent, the larger amount of DNA would contribute to enhance the effect of SN-38 in the tetraploid cells.

Sensitivity to anticancer drugs is often measured in terms of ability to induce apoptosis. Apoptosis in this study was measured with two different methods, both using the M30 antibody (Leers, 1999). One application was IHC-based to visualize and study the morphology, whereas flow cytometry was used as a more accurate quantitative method. The apoptotic response in the colon cancer cells showed time dependency, with longer exposure to SN-38 resulting in more apoptosis. After exposure to SN-38 for 4 hours, no apoptotic response could be detected in the colon cancer cells. After 24-hour exposure, the apoptotic rate is increased, and 48-hour exposure results in the highest frequency of apoptosis. Whether SN-38 induces apoptosis or not has been discussed. It has been reported that SN-38 induces apoptosis in testicular carcinoma cells (Ueno, 2002). It is also postulated that the status of cell-cycle-associated proteins such as p21 and p53 affects the result of SN-38 treatment. Wild type p53, with intact cell cycle checkpoints, induces long-term cell cycle arrest, i.e. cellular senescence (te Poele and Joel, 1999). In contrast to mutation of p53, which causes premature mitosis due to transient G2/M arrest, followed by mitotic catastrophe and finally apoptosis (Bunz, 1998; Magrini, 2002).

Similar effects are seen for p21, which is a downstream effector of p53. Intact p21 cells arrest permanently in G2 compared with mutated p21 cells, which after exposure to SN-38 respond with apoptosis (Motwani, 2001). The cell lines studied have a p53 mutation at amino residue 179, resulting in a shift His→Arg (Okamoto, 2000). As expected for p53 mutated cells, apoptosis was induced after SN-38 treatment in the three KM12 cell lines. Regarding the cell cycle distribution, a transient G2 arrest would be expected. KM12C and KM12SM both show G2 arrest. It is not, however, of a transient character, rather a constant level of cells. This might be due to cells forcing the M phase, despite potential DNA damage caused by the inactive p53. Opposite to a transient G2 arrest KM12 L4a cells accumulate in G2, like a permanent arrest. One explanation of this might be the short follow-up. The result might be different with a longer follow-up. The experiment could have been improved by exposing the cell cultures for a shorter period followed by withdrawing the old medium containing the remains of the drug. With respect to the total withdrawal of the drug, SN-38 has a rather short halftime and is also light-sensitive, suggesting that just smaller fractions of the active drug would be left after the long time exposure. However, it would be interesting to study the cells 3 weeks after exposure.

The protein expressions of topo-1, Bax and survivin were examined by western blot. The efficacy of SN-38 is thought to depend on the level of topo-1 (McLeod and Keith, 1996). This is favorable because tumor cells often have a higher expression of topo-1 than normal cells do, sensitizing them against SN-38 and protecting normal cells (Giovanella, 1989). In this study the topo-1 expression decreased in a reversed time-dependent manner, with lower expression compared with the untreated controls already after 4 h exposure and total absence of topo-1 expression after 48 h. This supports previous findings suggesting that topo-1 is a predictive factor for irinotecan treatment. In the intrinsic stimulated pathway of apoptosis pro-apoptotic Bax, a member of the Bcl-family, plays a central role (Kirkin, 2004). Bax translocated to the mitochondria promotes cyt *c* release, and downstream the activation of caspase-3 and -9 (Gross, 1998; Antonsson, 2000; Antonsson, 2001). In Paper II the Bax expression increased in KM12L4a after 24-hour and 48-hour treatment. Overexpression in head and neck squamous cell carcinoma and in erythroleukemia cells after treatment with SN-38 has been reported (Kobayashi, 1998; Guo B., 2000). Altogether these findings support the idea of Bax as a potential target against SN-38 sensitization. The therapeutic implication of survivin is broad. Overexpression of survivin is related to drug resistance (Longley and Johnston, 2005), and there is also evidence that upregulated survivin is a negative effector in irinotecan treatment (Pyrko et al). Notably we found that the SN-38 treatment itself seems to affect the survivin expression and that a low expression of the protein coincides with high apoptosis.

Paper IV

In this study, colon cancer cell lines KM12C, KM12SM and KM12L4a were treated with topo-1 inhibitor SN-38 to further elucidate how the gene expression is affected by treatment. SN-38, as an active metabolite of Topo-1 inhibitor irinotecan, binds to topo-1 and stabilizes thereby the cleavable complexes. In this way a multi-stage pathway is initiated, starting with the formation of DNA damage and ending with cell death. In Paper III, exposure of SN-38 to KM12C, KM12SM and KM12L4a caused S-phase/G2 arrest and apoptosis. Increased Bax expression and decreased Topo-1 expression were also detected (Wallin et al., 2008). In Paper IV, we further examined the gene expression profile of the same cell lines after treatment with SN-38, with the aim of identifying highly effected genes that could act as biomarkers or potential targets for drug sensitization.

Analyses of the functional groups of the genes showed that apoptosis-associated genes were highly affected by SN-38 in both groups of downregulated and upregulated genes. The majority of the genes (1036) were downregulated, with only 417 genes upregulated. Among the downregulated genes, *DOK6* was on the top of the gene list, a gene that interacts with proto-oncogene *RET*. In thyroid carcinoma xenografts, inhibition of RET significantly increased the apoptotic response (Petrangolini, 2006). *IBtk* was another downregulated gene, and it functions as an inhibitor to Btk and subsequently as an inhibitor of NFκB-driven transcription (Liu W., 2001). Proto-oncogene K-ras is frequently overexpressed in CRC and associated with a high proliferative state, among other things (Sun, 1998). After SN-38 treatment *K-ras* expression was downregulated although with low significance (Golub score < 3). Underexpression of *BIRC5* was detected, also with a Golub score < 3. Survivin, encoded by *BIRC5*, as a member of the IAP family, blocks the apoptotic process by inhibiting primarily caspase-3 and -7 and is proposed as a target for drug therapies due to its limited expression in tumor cells (Altieri, 2008). Even though K-ras and BIRC5 have Golub scores that indicate low significance, their altered expression may have influence the apoptotic response. Smaller changes in the genetic expression, might together result in a synergistic effect. Of the upregulated genes based on the Golub score, the top four genes *SGK*, *PHLDA2*, *IER3*, and *RhoB*, are all implicated in apoptosis. For example, *RhoB*, a member of the RAS superfamily and which functions as a tumor suppressor (Jiang, 2004), was highly ranked, as was *IER3,* which through the inhibitory effect of NFκB (Sebens Muerkoster, 2008) also acts as a tumor suppressor. In HeLa cells blockade of NFκB promotes apoptosis, following SN-38 treatment (Bottero, 2001). Further pro-apoptotic *Bax* was also found to be upregulated as well as *IκBB* (*NFκBIB*), an inhibitor of NFκB (Jacobs and Harrison, 1998). Upregulation of *Bax* after SN-38 treatment has been demonstrated earlier (Hayward, 2003; Souza, 2005), and overexpression of

protein Bax has also been favorable in the way it enhances the induction of apoptosis (Guo B., 2000). These findings of altered expression in the apoptosis-associated genes, strengthens SN-38 as a potent inducer of apoptosis.

Some genes were downregulated or upregulated after the SN-38 treatment, in the opposite manner to what would be expected. For example, *WT1* (*PAWR*) was downregulated, although it functions as a tumor suppressor in the way it represses transcription, and is an apoptosis inducer via Bak (Morrison, 2005).

Genes involved in cell proliferation and apoptosis seem to be affected by SN-38 in a positive manner, i.e. promoting cell cytotoxicity. The status of tumor suppressor p53 is widely implicated in the success of chemotherapy (Bossi and Sacchi, 2007), mainly through its role in apoptosis. The three cell lines, KM12C, KM12SM and KM12L4a, in this study are p53 mutated (Okamoto, 2000). It is postulated that two different events could occur after SN-38 treatment, depending on the p53 status. For p53 wild type cells, cellular senescence is induced via S/G2-arrest, which subsequently becomes permanent, compared with p53 mutated cells, which will suffer from mitotic catastrophe and apoptosis (Motwani, 2001; Magrini, 2002). This coincides with the results of altered expressions of apoptosis associated genes described here, but also with the result from Paper III (Wallin, 2008).

It is widely discussed whether the expression of *topo-1* is altered following SN-38 treatment. In this study no such evidence of change in RNA expression after SN-38 treatment could be found, and this was consistent with other findings (Hertzberg, 1989; Motwani, 2001; Souza, 2005). However, in our previous study, topo-1 protein expression was found to be downregulated following SN-38 treatment (Wallin, 2008). Changes in RNA expression do not necessarily have to have a direct impact on the protein expression. Possibly the deviant expression derived from post-transcriptional or translational alterations.

It is notably that the cell lines display similar gene expression profiles. The two metastatic derivatives, KM12SM and KM12L4a, were originally established from the parental cell line, KM12C. Although the ability to form metastasis may vary between the cell lines, the response to SN-38 is similar, indicating a non-significant influence of the metastasis related factors. In Paper II and Paper III different protein expression levels were detected depending on their metastatic potential (Wallin, 2008; Pfeifer, Manuscript), although in the current study all samples were analyzed together for the gene expression profile and not as individual samples. In other words, the exact expression of each cell line could not be detected. Therefore it is required that the expression of certain genes be analyzed for each specific cell line.

The present results indicate that the expression of the genes involved in cell proliferation and apoptosis was affected by SN-38 based on their RNA

expression. The impact of certain genes on CRC development needs to be further investigated. However the results could serve as basis for further studies to find targets for irinotecan treatment.

CONCLUSIONS

In this thesis the response to γ-radiation and SN-38 treatment on CRC have been investigated. We focused on studying apoptosis/apoptotic associated factors and the metastatic impact. Based on the results in Paper I-IV, the conclusions are:

- Strong expression of PRLs was found at the invasive margin, which acts as a frontier in the tumor development and progression. This supports the evidence for PRLs being involved in invasion.

- In the RT group strong expression was correlated to distant recurrence and poor survival. The PRL proteins may therefore serve as biomarkers predicting RT response.

- In absence of functional p53 the p73 isoforms seem to have a restoration effect. Lack of upregulation of ΔNp73, survivin and PRL-3 in KM12L4a probably explains the higher radiosensitivity.

- SN-38 affects both the G2 and the s-phase arrest, and it also induced apoptosis in colon cancer cell lines. Downregulation of anti-apoptotic survivin and upregulation of pro-apoptotic Bax probably affected the apoptotic inducement.

- Treatment with SN-38 altered the genetic profile of colon cancer cells *in vitro*. Several genes, e.g. *survivin, DOK6, RhoB* and *Bax*, are down- respectively upregulated in a favorable way promoting apoptosis.

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