Molecular Studies of Radiotherapy and Chemotherapy in Colorectal Cancer
To Elise, Tore and Alex
JASMINE EVERT

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Abstract


Colorectal cancer is a common malignancy, with more than 6000 new cases diagnosed each year in Sweden. The primary treatment is surgery, which is often combined with radiotherapy and/or chemotherapy in order to decrease the risk of recurrence. Both radiotherapy and chemotherapy are associated with side effects and there is significant variation in treatment response among patients. The aim of this thesis was to study molecular factors influencing the response to radiotherapy and chemotherapy.

The adaptor protein PINCH, thought to promote tumour progression, was studied in paper I. PINCH was expressed in stromal cells in and around tumours, and expression in normal mucosa was related to survival. PINCH expression was also related to outcome of chemotherapy. The p53 homologue p73 was studied in papers II and III. In paper II, a G4C14→A4T14 polymorphism of the p73 gene was investigated in rectal cancer patients with or without radiotherapy. It was found that the polymorphism could influence the outcome of radiotherapy. When combining the GC/GC genotype with wild-type p53 and low expression of survivin, the results were significant. In paper III, the p73 isoform ΔNp73β was found to increase cellular viability in colon cancer cells. In paper IV, the effects of the chemotherapeutic drug oxaliplatin, p53 and p73 status on the expression profile of miRNAs in colon cancer cells were studied. A number of miRNAs were up-or down-regulated in response to oxaliplatin, and p53 and p73 influenced this response.

Keywords: Colorectal cancer, Radiotherapy, Chemotherapy, p53, p73, PINCH, miRNAs.

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List of abbreviations

5-FU 5-Fluorouracil
Apaf-1 Apoptotic protease activating factor-1
APC Adenomatous polyposis coli
ATM Ataxia telangiectasia mutated
bp Base pair
BLAST Basic local alignment search tool
CCD Charge coupled device
Cdk Cyclin-dependent kinase
cDNA Complementary DNA
Chk Checkpoint kinase
CK18 Cytokeratin 18
CMV Cytomegalovirus
DAB Diaminobenzidine
DAPI Diamidino-2-phenylindole
DBD DNA-binding domain
DCC Deleted in colon cancer
DIABLO Direct inhibitor of apoptosis-binding protein with low pI
DISC Death-inducing signalling complex
DNA Deoxyribonucleic acid
dNTP Deoxyribonucleotide triphosphate
dsRNA Double-stranded RNA
EtBr Ethidium Bromide
ECL Enhanced chemiluminescence
ECM Extracellular matrix
EGFR Epidermal growth factor receptor
ELISA Enzyme-linked immunosorbent assay
FAP Familial adenomatous polyposis
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Gy</td>
<td>Gray</td>
</tr>
<tr>
<td>HNPCC</td>
<td>Hereditary non-polyposis colon cancer</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitor of apoptosis</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>ILK</td>
<td>Integrin-linked kinase</td>
</tr>
<tr>
<td>IRS-1</td>
<td>Insulin receptor substrate-1</td>
</tr>
<tr>
<td>Mdm2</td>
<td>Mouse double minute 2</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<td>MMR</td>
<td>Mismatch repair</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>MSI</td>
<td>Microsatellite instability</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotide</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>OD</td>
<td>Oligomerization domain</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PEST</td>
<td>Penicillin-streptomycin</td>
</tr>
<tr>
<td>PINCH</td>
<td>Particularly interesting new cystein-histidine-rich protein</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>SAM</td>
<td>Sterile alpha motif</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>Smac</td>
<td>Second mitochondria-derived activation of caspase</td>
</tr>
<tr>
<td>TA</td>
<td>Transactivation</td>
</tr>
<tr>
<td>TME</td>
<td>Total mesorectal excision</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumour node metastasis</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>XTT</td>
<td>2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carbox-anilide</td>
</tr>
</tbody>
</table>
List of papers

This thesis is based on the following original papers, which will be referred to in the text by their roman numerals (I-IV):

   Impact of PINCH expression on survival in colorectal cancer patients
   *BMC Cancer* (2011) 11:103

II. Lööf (Evert) J, Pfeifer D, Adell G, Sun XF
    Significance of an exon 2 G4C14-to-A4T14 polymorphism in the p73 gene on survival in rectal cancer patients with or without preoperative radiotherapy

III. Lööf (Evert) J, Pfeifer D, Ding Z, Sun XF, Zhang H
    Effects of ΔNp73β on cisplatin treatment in colon cancer cells

IV. Evert J, Pathak S, Sun XF, Zhang H
    Modification of microRNA expression profiles by oxaliplatin, p53 and p73 in human colon cancer cells in vitro
    *Submitted*

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Paper II © 2009 Elsevier, Radiotherapy and Oncology.
Introduction

Cancer is a complex disease that can affect almost every organ or tissue in the body. Cancer cells have acquired a number of traits enabling the uncontrolled proliferation that characterizes cancer cells. These traits are called the Hallmarks of cancer [1] and include self-sufficiency in growth signals, insensitivity to anti-growth signals, evading programmed cell death (apoptosis), limitless replicative potential, the ability to grow new blood vessels in the tumour (angiogenesis) and the ability to invade surrounding tissue and metastasise to distant sites in the body. Another two traits of cancer cells are emerging as hallmarks of cancer: deregulation of cellular energetics and avoiding immune destruction [1]. The process in which a cell acquires these hallmarks is multistep, and includes the accumulation of genetic alterations in the cell. These alterations often occur in proto-oncogenes, tumour suppressor genes or genes involved in DNA-repair [2].

Colorectal cancer

Colorectal cancer is a common malignancy with more than 1.2 million new cases diagnosed worldwide in 2008, and it is ranked the third most common cancer in men and second in women [3]. The mortality rate is approximately 50%, making it a common cause of cancer-related death. In Sweden colorectal cancer is the third most common cancer in both men and women, with more than 6400 new cases reported in 2013 [4].

Colorectal cancer comprises cancer of the colon and the rectum. The intestinal wall is structured into four layers: the mucosa, submucosa, muscularis propria, and serosa (Figure 1). Tumours arise in the mucosa, the innermost layer.

![Figure 1. Colon, rectum and the layers of the intestinal wall](image)
Aetiology and risk factors
The aetiology of colorectal cancer is multifactorial, consisting of environmental as well as hereditary factors. The vast majority of all cases of colorectal cancer can be attributed to sporadic disease, in which there is no apparent predisposing aetiology. The remaining cases are accounted for by hereditary or familial disease and inflammatory bowel disease, including Chrons’ disease and ulcerative colitis, which increases the risk of colorectal cancer [5]. A family history of colorectal cancer in first-degree relatives presents as a risk factor, but the majority of familial disease has no clearly identifiable genetic aetiology. Some familial cases of colorectal cancer consist of well-described hereditary syndromes, accounting for 1-5% of all colorectal cancer cases. These include familiar adenomatous polyposis (FAP) and hereditary non-polyposis colon cancer (HNPPCC) [6]. FAP is associated with a 100% risk of developing colorectal cancer and is characterized by the development of multiple colonic polyps early in life, caused by a germline mutation in the tumour suppressor gene APC (Adenomatous polyposis coli). HNPPCC is caused by germline mutations in mismatch repair (MMR) genes and is associated with an 80% lifetime risk of developing colorectal cancer.

There are considerable differences in colorectal cancer incidence between different parts of the world. The highest incidence rates are found in Australia and New Zealand, Europe and North America, whereas the lowest incidence rates are found in Africa and South-Central Asia [3]. This difference is mainly attributed to lifestyle factors, and the Western lifestyle is regarded as the main explanation [7]. A high intake of red and processed meat, smoking, excessive alcohol consumption, physical inactivity and excess body weight are risk factors for colorectal cancer [8-10].

Carcinogenesis
Colorectal cancer most commonly arises from benign, adenomatous polyps lining the bowel wall. The carcinogenesis is a multistep process beginning in a stem cell in the normal mucosa. A series of mutations is required for a normal cell to be transformed into a tumour cell, and these mutations occur in oncogenes, tumour suppressor genes and mismatch repair (MMR) genes. Oncogenes normally stimulate cell proliferation and decrease cell cycle arrest and apoptosis, while tumour suppressor genes do the opposite [11]. MMR-genes repair mistakes made during DNA replication and damages induced by mutagens. Impaired DNA mismatch
repair result in deficient repair of spontaneous replication errors. Mutations in the MMR-genes therefore increase the risk of alterations in other genes [11].

An activating mutation in an oncogene or an inactivating mutation in a tumour suppressor gene can cause a disorder in cell replication and renewal, resulting in for example enhanced cellular replication or inhibition of apoptotic cell death. In the colon and rectum, this could lead to the development of dysplastic cellular changes and the growth of adenomatous polyps called adenomas, above the surrounding mucosa. The development of these adenomas into a malignant carcinoma is referred to as the adenoma-carcinoma sequence (Figure 2), in which a number of sequentially accumulated mutations eventually result in an adenocarcinoma [6, 12]. These tumours are called chromosomal instable tumours, and account for approximately 85% of sporadic colorectal cancers. The earliest event is commonly the inactivation of the tumour suppressor gene APC, leading to the transformation of the normal mucosa into an early adenoma. This is typically followed by mutational activation of the oncogene K-RAS, causing the adenoma to further grow and progress. Mutation of the tumour suppressor p53 appears at a later stage in tumour development and is associated with the malignant transformation from adenoma to carcinoma. DCC (Deleted in colon cancer) and SMAD4 are also tumour suppressors often missing in colorectal cancer. The remaining 15% of sporadic colorectal cancers are microsatellite instable (MSI) tumours, which are caused by mutations in MMR genes [13].

![Figure 2. The adenoma-carcinoma sequence (Adopted from Fearon and Vogelstein, 1990)]
Staging

The prognosis of a cancer patient is affected by multiple factors including cancer type, tumour spread, presence of metastases, and grade of differentiation (resemblance with normal tissue). In colorectal cancer, pathologic stage represents one of the most important prognostic factors. Colorectal cancer is categorized according to the tumour node metastasis (TNM) or the Dukes staging systems (Table 1). The TNM system offers advantages over the Dukes system and is now the most widely used. The TNM system is based on the extent of the tumour (T), the extent of spread to the lymph nodes (N), and the presence of distant metastases (M) [14]. A number added to each letter indicates the extent of the tumour and the spread. Different TNM combinations correspond to one of five stages, referred to as stage grouping. There are five stages expressed in roman numerals, from stage 0 to stage IV. The Dukes system, presented in 1932 by the English pathologist Dukes, includes four stages: A, B, C and D, describing the depth of tumour invasion, extent of regional lymph node involvement and presence of distant metastases.

Table 1. The TNM and Dukes staging systems in colorectal cancer.

<table>
<thead>
<tr>
<th>TNM staging</th>
<th>Description</th>
<th>Dukes stage</th>
<th>5-years survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Tis, NO, M0</td>
<td>Tis Carcinoma-in-situ, tumour confined to mucosa</td>
<td></td>
<td>&gt;95%</td>
</tr>
<tr>
<td>I T1, NO, M0 T2, NO, M0</td>
<td>T1 T2 Tumour invades submucosa Tumour invades muscularis propria</td>
<td>A</td>
<td>80-95%</td>
</tr>
<tr>
<td>II A T3, NO, M0</td>
<td>T3 Tumour invades through muscularis propria into pericoleorectal structures</td>
<td>B</td>
<td>60-80%</td>
</tr>
<tr>
<td>II B T4, NO, M0</td>
<td>T4 Tumour directly invades other organs, structures or perforates the visceral peritoneum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III A T1, T2, N1, M0 T3, T4, N1, M0</td>
<td>N1 N2 Metastases in one to three regional lymph nodes Metastases in four or more regional lymph nodes</td>
<td>C</td>
<td>30-55%</td>
</tr>
<tr>
<td>III B Any T, N2, M0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III C Any T, Any N, M1</td>
<td>M1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV Any T, Any N, M1</td>
<td>M1 Distant metastases</td>
<td>D</td>
<td>&lt;10%</td>
</tr>
</tbody>
</table>
Treatment
The primary treatment for colorectal cancer is surgery, which is often combined with chemotherapy or radiotherapy to reduce the risk of recurrent disease. Patients that are not candidates for curative surgery can be given palliative chemotherapy or radiotherapy to prolong survival and decrease suffering [15]. If surgery is possible, the affected colonic or rectal segment should be removed with a certain tumour-free margin, together with lymph nodes associated with the tumour. The removal and examination of lymph nodes is important both for preventing recurrence and for deciding adjuvant treatment [16]. The treatment of tumours situated in the rectum is somewhat different from that of tumours in the colon. Because of the proximity with the anal verge, tumour-free resection margins may be difficult to achieve while preserving the function of the anal sphincter. A low rectal cancer, situated close to the anus, therefore commonly requires an abdominoperineal resection or rectal amputation. This leaves the patient with a permanent colostomy, as this procedure requires a total excision of the rectum including the anal canal and the sphincter. The standard surgical treatment for tumours located higher in the rectum is an anterior total mesorectal excision (TME) [17], sparing anal sphincter function.

Most patients with primary resectable rectal cancer are treated with radiotherapy in order to decrease the risk of recurrence. The ionising radiation damages the DNA in the tumour cells by causing single- or double strand brakes. As double strand brakes are lethal to the cell, radiation can cause cell death through one of two mechanisms: apoptosis or necrosis [18]. While necrosis occurs due to severe cell damage in an uncontrolled manner that includes lysis of the cell and an inflammatory response, apoptosis is a highly controlled process. Apoptosis is also called programmed cell death and follows a well-controlled pathway with minimal damage to the surrounding tissue. Radiotherapy can be administered before or after surgery, at higher doses during a short period of time, or at lower doses during longer time [19]. The Swedish Rectal Cancer Trial performed in 1987-1990, was the first study to show that short-term (5X5 Gy) preoperative radiotherapy significantly improves overall survival, and is currently the treatment of choice in Sweden [20]. Adjuvant chemotherapy is often given after surgery to reduce the risk of recurrent disease. Chemotherapeutic agents are cytotoxic, primarily targeting fast dividing cells, such as cancer cells. The most commonly used drug for treatment of colorectal cancer is 5-Fluorouracil (5-FU), an
inhibitor of the enzyme thymidylate synthase, which is involved in the nucleotide synthesis [21]. In order to enhance the effectiveness, leucovorin (reduced folic acid) is usually given together with 5-FU [22]. 5-FU is often combined with oxaliplatin or irinotecan [23, 24], cytotoxic agents that inhibit DNA replication. Oxaliplatin, a platinum-based alkylating compound, binds the DNA and forms DNA adducts. Irinotecan inhibits the enzyme topoisomerase I. In addition, biological agents such as monoclonal antibodies targeting the vascular endothelial growth factor (VEGF) (Bevacizumab) and epidermal growth factor receptor (EGFR) (Cetuximab and Panitumumab) are successfully being used in the treatment of colorectal cancer [25].

Both radiotherapy and chemotherapy are associated with side effects and there is significant variation in the response to the treatment even among patients at the same tumour stage. Predictive and prognostic markers in colorectal cancer patients have been the subjects of intense research. The determination of prognosis predominantly relies on the histopathological examination, although there are certainly other factors influencing survival. Approaches are being made to improve prognostic methods, including analysing additional histopathological factors and molecular and genetic markers. Although these markers are promising they are not yet routinely used. Potential markers include, for example, allelic imbalances, chromosomal instability, expression of oncogenes, loss of tumour suppressor genes, markers of proliferation, angiogenesis, inflammation and cell adhesion as well as genes involved in the response to chemotherapy and radiotherapy.
Cell Cycle
The series of events leading up to the duplication of one cell into two identical daughter cells is called the cell cycle (Figure 3). The cell cycle of eukaryotic cells consists of the interphase and the mitotic (M) phase. The interphase is subdivided into three different phases: G1, S and G2 phase. In G1, the cell grows and prepares for DNA replication, which occurs during the DNA synthesis (S) phase. During the G2 phase, the cell continues to grow and prepare for the cell division in the M phase. The cells may continue to divide or enter a phase called G0, a non-proliferative, resting phase. The cell cycle is highly regulated and contains several checkpoints that prevent the cell cycle to proceed unless certain requirements are met. Two important checkpoints are located in the G1 and G2 phase. The G1 checkpoint, known as the restriction point, prevents entry into the S-phase if there is DNA damage, and the G2 checkpoint prevents entry into the M-phase in case the chromosomes are faulty [26]. Cytotoxic agents and radiation that causes DNA damage activates the checkpoints, thereby causing cell cycle arrest during which DNA repair may be allowed [18]. A dysregulation of the cell cycle may lead to tumour formation. Loss of tumour suppressors such as p53 and Rb due to mutation could cause a cell to divide uncontrollably.
Apoptosis

Cell death is generally divided into two main types, necrosis and apoptosis, both of which may be induced by radiation and anti-cancer agents. Necrosis is caused by severe cell damage and is characterised by loss of membrane integrity, cell swelling and eventually cell lysis. As the cell contents are released in the surrounding tissue, an inflammatory response is induced. Unlike necrosis, apoptosis is a highly controlled process. Apoptosis was first described in 1972, and the term is derived from the Greek word for “falling off”, reflecting the morphological changes of dying cells [27]. The apoptotic cell is characterised by shrinkage, membrane blebbing, condensation of the chromosomes, DNA fragmentation and eventually the fragmentation of the cell into apoptotic bodies that are engulfed by macrophages, preventing an inflammatory response.

Apoptosis can be initiated through one of two pathways: the extrinsic or the intrinsic (Figure 4). The extrinsic pathway begins with the binding of extracellular ligands, including Fas, Tumour Necrosis Factor-α (TNF-α) or TNF-related apoptosis inducing factor (TRAIL), to their respective receptors in the cell membrane. Following ligand binding, adaptor proteins are recruited to the intracellular domains of the receptors, forming the death inducing signalling complex (DISC) [28, 29]. DISC in turn activates caspases, a family of cysteine proteases [30]. The caspases activated by DISC, caspases 8 and 10, are called initiator caspases. Once they are activated, they continue to cleave and thereby activate another group of caspases called effector caspases. These in turn cleave a number of vital proteins, leading to the degradation of the cell [31].

The intrinsic pathway is initiated as a result of intracellular stress such as DNA damage, oxidative stress or oncogene activation. As the process is initiated, the mitochondrial membrane becomes permeabilised, leading to an efflux of cytochrome C [32]. Cytochrome C forms an apoptosome together with protease activating factor-1 (Apaf-1) and procaspase-9 [30]. The apoptosome activates effector caspases that degrade protein components in the cell. The intrinsic and the extrinsic pathway converge at the activation of the effector caspases [32]. The activity of the effector caspases is partly regulated by a family of proteins called the inhibitor of apoptosis proteins (IAPs) [33]. These inhibit the activity of the effector caspases, thereby blocking apoptosis. The IAPs in turn are antagonised by proteins called Smac/DIABLO that are released from the mitochondria in response to apoptotic stimuli [34, 35].
One of the Hallmarks of cancer is evading apoptosis, and alterations affecting damage-induced apoptosis could lead to cancer cells being resistant to DNA damaging treatment such as chemo- or radiotherapy. The apoptotic process is highly regulated and is dependent on a number of pro- and anti-apoptotic proteins. The Bcl-2 family of proteins regulates cytochrome C release from the mitochondria, and one strategy for tumour cells involves modifications of this protein family, such as loss of pro-apoptotic Bax [36] and Bak [37]. Another strategy to avoid apoptosis is loss of the tumour suppressor p53, which is central in the induction of apoptosis following DNA damage.

Figure 4. The extrinsic and intrinsic pathways of apoptosis
The p53 family

The p53 family consists of p53, p63 and p73. Members of the p53-family share three major functional domains: the N-terminal transactivation domain, the central core sequence specific DNA-binding domain, and the C-terminal oligomerization domain. In addition, p63 and p73 contain a sterile alpha motif (SAM) domain [38] (Figure 5).

Figure 5. The p53-family members p53, p63 and p73 contain a transactivation (TA), DNA-binding (DBD) and oligomerization (OD) domain. In addition, p63 and p73 contain a sterile alpha motif (SAM) domain. The p63 and p73 genes contain two promoters, P1 and P2, giving rise to the full-length isoforms TAp63 and TAp73 and the truncated isoforms ΔNp63 and ΔNp73.

p53

p53, “the guardian of the genome” [39], is a tumour suppressor gene that is commonly mutated in human cancers. In colorectal cancer, mutations of the p53 gene, TP53, can be identified in approximately 50% of all cases [40]. In response to cellular stresses such as DNA damage, p53 promotes the transcription of genes involved in cell cycle arrest or apoptosis. The half-life of p53 is short, keeping protein levels in the nucleus low if there is no DNA damage. p53 induces the expression of mouse double minute 2 (mdm2) [41], which in turn promotes the rapid degradation of p53 [42]. DNA damage induces the Ataxia telangiectasia mutated (ATM) kinase to phosphorylate and stabilise p53 [43], which then accumulates rapidly and causes cell cycle arrest or apoptosis. p53 binds the DNA and induces the
transcription of p21, an inhibitor of cyclin dependent kinases (CDKs). p21 prevents the cell from entering the S-phase, allowing DNA repair to take place [26]. If the damage is repaired, the cell will be able to complete the cell cycle. When damage is too severe, p53 promotes apoptosis by inducing the transcription of pro-apoptotic genes such as Bax, Noxa and Puma [44-46]. Given the central role of p53 in the response to DNA damage, a loss of function of this protein may result in decreased sensitivity to anti-cancer treatment. In colorectal cancer, loss of p53 has been associated with resistance to radiotherapy and chemotherapy [47, 48].

### p63

The p63 gene, TP63, was identified in 1998 as one of two p53 homologues [49]. Phylogenetic analysis suggests that p63 might be the evolutionary predecessor of both p53 and p73. The p63 gene contains two promoters, resulting in different isoforms of the protein: the full-length protein, TAp63, and a truncated variant termed ΔNp63, lacking the transactivating capability of TAp63. Further, alternative splicing gives rise to C-terminal variation. p63 has a central role in epithelial development [50].

### p73

The p73 gene, TP73, was identified in 1997. Being structurally and functionally homologous to p53, p73 can activate the transcription of p53-responsive genes and induce cell cycle arrest or apoptosis in a p53-like manner [51]. p73 expression is kept low under normal physiological conditions and is, like p53, induced to be stabilised at the protein level in response to various DNA-damaging stimuli [52]. It has been shown that ATM induces the tyrosine kinase c-abl, which phosphorylates and activates p73 in response to DNA-damaging agents such as γ-radiation and cisplatin [53, 54]. p73 can also be activated by checkpoint kinases Chk1 and Chk2 through stabilising of the transcription factor E2F1, which induces the expression of TAp73 [55].

p73 is expressed as various isoforms differing both C- and N-terminally. There are at least six different C-terminal isoforms (α, β, γ, δ, ε and ζ), due to alternative splicing of the primary transcript [56]. Like p63, the use of alternative promoters results in N-terminal variation (Figure 5). The P1 promoter located upstream of exon 1, gives rise to the full-length isoforms of the p73 protein, TAp73, containing an intact transactivation
domain. An alternative promoter, P2, located in intron 3, gives rise to N-terminally truncated isoforms termed ΔNp73 [57]. These isoforms lack the transactivation domain, and are therefore incapable of inducing cell cycle arrest or apoptosis [58]. Interestingly, the ΔNp73 isoforms inhibit the function of TAp73 and p53, either by oligomerizing with the full-length protein or by binding p53/TAp73 responsive elements, displacing p53 and TAp73 from the DNA binding site [58]. Further, both p53 and TAp73 induce the expression of ΔNp73 through a p53/TAp73 responsive element within the ΔNp73 promoter region, creating a negative feedback loop that regulates the function of p53 and TAp73 [59].

The p73 gene is located on chromosome 1p36, a region frequently deleted in a variety of cancers [38]. It has been speculated that p73, like p53, is a tumour suppressor. However, unlike mice lacking functional p53, p73-deficient mice do not develop spontaneous tumours [57], and p73 is rarely mutated in primary tumours [60]. Rather, p73 is overexpressed in various tumour types including colorectal cancer, suggesting an oncogenic role for p73 [61, 62]. The oncogenic properties of p73 are attributed to ΔNp73, because of the dominant negative behaviour towards p53 and TAp73. Further, ΔNp73 is involved in development of the brain in mouse, where it protects the neuronal cells from apoptosis [63]. Furthermore, knock-out mice lacking only TAp73 are prone to developing tumours [64], supporting the idea that TAp73 has tumour suppressor functions while ΔNp73 is anti-apoptotic.
PINCH

PINCH (Particularly interesting new cystein-histidine-rich protein), an adaptor protein belonging to the LIM-family of proteins, was first identified in 1994 [65]. PINCH directly associates with two proteins: ILK, (Integrin-linked kinase) [66], and Nck-2 [67]. ILK is a protein kinase that interacts with the cytoplasmic domain of integrin-β1, thereby regulating integrin-mediated cell signalling and adhesion [68]. Integrins are the main receptors of extracellular matrix (ECM) proteins, and cell-ECM-signalling via integrins is essential for embryonic development, proliferation, survival, adhesion, differentiation and migration [69]. ILK is a constituent of integrin-mediated cell-matrix focal adhesions [70], structures that mediate cell adhesion and signal transduction between the ECM and the intracellular compartment. Further, ILK regulates the assembly of the ECM component fibronectin in a process that is dependent on PINCH [71]. PINCH and ILK have been shown to be indispensible for proper control of cell shape change, spreading, and motility. Further, PINCH and ILK regulate the PKB/Akt signalling pathway, which transmits extracellular survival signals to downstream effectors, including caspasases [72].

The adaptor protein Nck-2 recognises several key components of growth factor receptor kinase-signalling pathways, including EGF (Epidermal growth factor) receptors, PDGF (Platelet derived growth factor)-receptor-β and IRS-1 (Insulin receptor substrate-1) [67]. It has been shown that ILK together with PINCH is capable of forming a multiprotein complex with Nck-2, indicating that PINCH could provide a connection between the growth factor receptor signalling pathways and cell adhesion receptor integrin-mediated pathways.

PINCH has been shown to be up-regulated in tumour-associated stroma, while in normal tissue PINCH expression is minimal [73]. The increased expression is especially prominent at the tumour invasive margin of common carcinomas. The tumour-associated stroma consists of fibroblasts, myofibroblasts, smooth muscle cells, vascular elements, inflammatory cells and extracellular matrix and is important in facilitating cancer growth and invasion. Since ILK and PINCH regulate the assembly of fibronectin, an increase in PINCH expression could be associated with an enhanced fibronectin matrix assembly, providing an appropriate surface for tumour cells to migrate on [73].
miRNAs

MicroRNAs (miRNAs) were first discovered in 1993 by Ambros and colleagues. They are small non-coding RNAs of 18-24 nucleotides that regulate gene expression post-transcriptionally [74], and are involved in various biological processes including cell proliferation, differentiation and apoptosis [75]. More than 2500 human miRNAs have been identified to date, and it is estimated that around 30% of all protein-encoding genes are regulated by miRNAs [76, 77].

miRNAs are transcribed as pri-miRNAs, which are cleaved in the nucleus by an endonuclease called Drosha, forming a 60-70 nt stem-loop structure known as the miRNA precursor (pre-miRNA). The pre-miRNA is transported by ran-GTP and exportin-5 to the cytosol, where it is further processed by the enzyme Dicer, forming a miRNA duplex. After the separation of the duplex, the mature miRNA is incorporated in the RNA-silencing complex (RISC), which regulates gene expression either by translational repression [78, 79] or endonucleolytic cleavage of the mRNA target [80, 81]. A perfect match between the miRNA sequence and the mRNA target generally triggers endonucleolytic cleavage, while a non-perfect match commonly promotes translational repression [82] (Figure 6).

Half of all miRNA genes are located in cancer-associated genomic regions or fragile sites [83] and altered miRNA expression has been found in all human tumours, implicating miRNAs in tumourigenesis. Further, an increasing body of evidence suggests that miRNAs play an important role in modulating the chemosensitivity and chemoresistance of tumour cells [84]. Each miRNA has the ability to regulate hundreds of target genes, including oncogenes and tumour suppressors. The influence of miRNAs on these multiple mRNA targets may impact the cellular response to anticancer agents by regulation of survival and apoptotic pathways, specific drug targets, DNA repair systems or drug transport and metabolism.
Figure 6. miRNA biogenesis
Aims

The general aim of this study was to investigate prognostic and predictive molecular factors in colorectal cancer.

Specific aims:

- Investigate the relationship of PINCH protein expression with survival and clinicopathological variables in colorectal cancer patients.

- Study the effect of a G4C14-to-A4T14 polymorphism in the p73 gene on survival of rectal cancer patients treated with surgery alone or in combination with preoperative radiotherapy.

- Investigate the role of the p73 isoform ΔNp73β in the response to the chemotherapeutic drug cisplatin in colon cancer cells by overexpressing ΔNp73β in p53 wild type and p53 mutant colon cancer cell lines.

- Investigate the effects of the chemotherapeutic drug oxaliplatin, p53 and p73 status on the expression profile of miRNAs in colon cancer cells.
Materials and Methods

Colorectal cancer patients (Papers I and II)

In papers I and II of this thesis, patient data was obtained from surgical and pathological records. Survival data was obtained from the Cause of Death registry, provided by the Swedish National Board of Health and Welfare (Socialstyrelsen). All patients have given informed consent for the material to be used for scientific research. The studies were approved by the local Human Research Ethics Committee.

In paper I, immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue sections from 251 randomly selected patients with primary colorectal adenocarcinoma. The median age of the patients was 69 years (range 25-94 years). Tumour differentiation was graded as good, moderate, poor, or mucinous (including signet-ring cell carcinomas), and inflammatory infiltration was graded as weak, moderate or strong. Necrosis was graded as <10% and ≥ 10%. All patients underwent surgical resection at Linköping University Hospital (Linköping, Sweden) or Vrinnevi Hospital (Norrköping, Sweden), during the time period of 1973 to 2001. After surgery the patients were considered to have adjuvant chemotherapy, which was given to 27 patients. The main indication for adjuvant treatment was radically resected stage II or III tumours with additional risk factors (i.e. vascular invasion and poor differentiation) in colon cancer. Also, one rectal cancer patient with a stage III tumour and additional risk factors was included. Depending on various study protocols active at each time, the drugs and administration schedule differed.

In paper II, DNA was extracted from formalin-fixed paraffin-embedded surgical specimens from 138 rectal cancer patients participating in a randomized clinical trial of preoperative radiotherapy between 1987 and 1990 [20]. Surgical specimens were obtained by either rectal amputation or anterior resection. The mean age at diagnosis was 66 years (range 38–85). The mean follow-up time was 86 months (range 0–193). Sixty-five patients were randomized to preoperative radiotherapy, receiving 25 Gy in 5 fractions over a median of 6 days (range 5–12). Surgery was performed after a median of 3 days (range 1–13) after radiotherapy. Seventy-three patients had surgery alone. The data on the expression of p53, p73 and survivin was taken from previous studies at our laboratory [47, 85, 86].
Colon cancer cell lines

**HCT116 (Papers III and IV)**

The human colon cancer cell line HCT116, with wild-type p53 (HCT116\(^{p53^+}\)), and it’s p53-null derivative (HCT116\(^{p53^-}\)), was a kind gift from Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD). Both alleles of p53 have been targeted by homologous recombination in HCT116\(^{p53^-}\), resulting in a truncated protein lacking 40 amino acid residues [87]. The HCT116\(^{p53^-}\) cells do not express detectable wild-type p53 and are considered functionally p53-null. The HCT116 cell line is MMR-deficient.

The cells were maintained in McCoy’s 5A medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS (GIBCO, Invitrogen, Carlsbad, CA), 1.5 mM L-glutamine (GIBCO) and 1X PEST (GIBCO) at 37°C in a 5% CO\(_2\) incubator.

**HT29 (Paper III)**

The human colon cancer cell line HT29 harbours a mutation in the p53 gene, resulting in an Arg→His substitution of codon 273 and over-expression of the protein. The HT29 cells are MMR-proficient. The cells were maintained in McCoy’s 5A medium (Sigma-Aldrich) supplemented with 10% FBS (GIBCO), 1.5 mM L-glutamine (GIBCO) and 1X PEST (GIBCO) at 37°C in a 5% CO\(_2\) incubator.

**Cell transfections**

**cDNA (Paper III)**

In paper III, the ∆Np73β protein was overexpressed in the HCT116 and HT29 cell lines. The cells were transfected with a pCMV6-XL5 vector (OriGene, Rockville, MD) containing transfection-ready cDNA for ∆Np73β. The vector contains the human cytomegalovirus (CMV) promoter, which drives constitutive expression of the ∆Np73β cDNA insert. A pCMV6-XL5 vector lacking the cDNA insert was used as a negative control. The cells were treated with various concentrations of cisplatin (Sigma–Aldrich) 24 h after transfection. ∆Np73β overexpression was confirmed using western blot.
siRNA (Paper IV)
The term RNA interference (RNAi) is used to describe the process in which double stranded RNA (dsRNA) silences gene expression. This system, which is normally occurring in eukaryotic cells, was discovered by Andrew Fire and Craig Mello [88], for which they were awarded the Nobel price in 2006. In this process, dsRNA is cleaved by the endonuclease Dicer into small interfering RNAs (siRNA), 21-23 nucleotides in length, which become integrated in the RNAi-induced silencing complex (RISC). This complex guides the siRNA to a complementary mRNA sequence that is then cleaved and degraded, preventing the translation of the mRNA into protein [89]. siRNA technology is widely used as a tool for specific suppression of gene expression.

In this study, 21 bp dsRNA oligonucleotides (Invitrogen, Paisley, UK) homologous to 5′-specific TAp73 sequences absent from the ΔNp73 sequence were used to specifically knock-down TAp73 expression in HCT116p53+/+ and HCT116p53-/- cells. A non-silencing siRNA was used as a negative control. The siRNA sequences were subjected to BLAST search to confirm the absence of homology to any additional known coding sequences of the human genome. The colon cancer cells were reversely transfected with siRNA using Lipofectamine® RNAiMAX (Invitrogen) according to the manufacturer’s instructions. The cells were allowed to recover for 24 hours prior to oxaliplatin treatment. TAp73 protein expression became markedly reduced after siRNA gene knockdown, as determined by western blot analysis.

Treatments

Cisplatin (Paper III)
Cisplatin (cis-diammine-dichloro-platinum) is a platinum-based DNA-damaging drug that interacts with DNA, resulting in the formation of DNA adducts, primarily intrastrand crosslinks [90]. Subsequently, it induces DNA damage recognition proteins to signal to downstream effectors such as p53, resulting in cell cycle arrest and apoptosis. The colon cancer cells were treated with increasing concentrations of cisplatin and cellular viability was determined after 48 and 72 hours. Final concentrations and incubation times for subsequent experiments were 10 and 20 μM and 48 and 72 hours.
Oxaliplatin (Paper IV)
Oxaliplatin, a third generation diaminocyclohexane platinum compound, has been shown effective in the treatment of colorectal cancer [91]. As cisplatin, it damages the DNA by forming DNA adducts. Oxaliplatin was used at a concentration of 2 µM to treat colon cancer cells for 48 hours before harvesting the cells for protein- and RNA extraction.

Polymorphism genotyping

DNA extraction
In paper II, DNA was extracted from 50 µM paraffin-embedded tissue sections of normal lymph nodes, normal mucosa and tumour from rectal cancer patients. The extraction was performed using the Gentra Puregene Tissue Kit (Qiagen, Minneapolis MN) according to the manufacturer’s instructions. Briefly, after dissolving the paraffin, the tissue was cut into small pieces and digested in a cell lysis solution with Proteinase K at 55°C until completely dissolved, after which RNase A solution was added. Protein was precipitated using a protein precipitation solution and then the samples were centrifuged. DNA was precipitated from the supernatant using isopropanol and ethanol. The resulting dried DNA pellet was hydrated in a DNA hydration solution. The concentration and purity of the DNA was measured spectrophotometrically.

PCR
The polymerase chain reaction (PCR) is a commonly used technique to amplify a specific DNA sequence. It was invented in 1983 by Kary Mullis, for which he received the Nobel price in chemistry 1993. The PCR technique utilizes thermo stable Taq polymerase, deoxynucleotide triphosphates (dNTPs) and oligonucleotide primers complementary to the 3’ ends of each of the sense and anti-sense strand of the specific DNA sequence. The method is based on thermal cycling, and usually 20-35 cycles are performed. Each cycle consists of the following three steps: denaturation, annealing and elongation. During denaturation, the temperature is raised to 94-96°C, at which the double stranded DNA is separated into single strands. In the following annealing step, temperature is lowered to 50-65°C, allowing the primers to bind their complementary sequences on each side of the target sequence. Next, the temperature is raised to 72°C, which is the ideal working temperature for the Taq polymerase. The polymerase uses dNTPs to elongate the primers on the
single stranded template DNA, resulting in the synthesis of new DNA strands complementary to the template strands. Each newly synthesised strand of DNA becomes a template for further cycles, and the number of copies of the DNA target region increases exponentially with each cycle. The PCR product can be separated according to size with an agarose gel electrophoresis. Shorter fragments will move faster through the gel than longer. The result is visualised by adding ethidium bromide (EtBr) to the gel. The EtBr binds to the DNA and fluoresces under ultraviolet light.

In study II, the PCR technique was utilized to screen for a G4C14→A4T14 polymorphism in the p73 gene. We used confronting two-pair primers, which means that two pairs of primers were added in the same PCR reaction. One primer pair (F1 and R1) amplifies the A4T14 allele, producing a 270 bp fragment. The other primer pair (F2 and R2) amplifies the G4C14 allele, producing a 193 bp fragment. Primers F1 and R2 also produce a 428-bp fragment, common to all PCR runs irrespective of genotype. An example of the genotyping is seen in figure 7.

![Figure 7: Detection of the p73 G4C14→A4T14 polymorphism with PCR and confronting two-pair primers. Lanes 1 and 8: 100 bp ladder; Lanes 2–5: GC/AT and lanes 6–7: GC/GC. The F1 and R2 primer product is clearly visible in lanes 4–5.](image-url)
Immunological protein detection

Immunohistochemistry
Immunohistochemistry (IHC) allows the detection of specific proteins in a tissue section by using antibodies that bind to the protein of interest. The antibody-protein complex is usually visualised by using secondary antibodies conjugated to an enzyme that catalyses a colour-producing reaction, alternatively a fluorophore.

In paper I, IHC was used to detect the protein PINCH in tissue sections of normal mucosa, primary tumour and lymph node metastasis from colorectal cancer patients. Briefly, after deparaffinising the tissue sections, they were boiled in a high pressure cooker in order to unmask hidden epitopes. Endogenous peroxidase activity was blocked using an H₂O₂-methanol solution. In order to avoid non-specific antibody binding the sections were incubated with a protein block solution. Then the primary antibody, a polyclonal rabbit anti-PINCH antibody (Rockland Laboratories, Gilbertsville, PA), was applied. After incubation and washing, the secondary antibody, an anti-rabbit/mouse polymeric horseradish peroxidase (HRP) conjugate (Dako, Glostrup, Denmark) was added to the sections. The peroxidase reaction was performed using 3,3′-diaminobenzidine chromogene (DAB) (Dako). The HRP catalyses the oxidation of DAB, producing a dark-brown colour. The sections were counterstained with haematoxylin. Finally, the slides were microscopically investigated and scored independently by two investigators without any knowledge of the clinicopathological data.

Western blotting
Western blotting, or immunoblotting, is a widely used technique for determining the relative amount of a protein in a tissue or cell sample using specific antibodies. The first step of a western blot is separating the proteins of a cell lysate or tissue homogenate by gel electrophoresis, usually sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins are separated according to size, as smaller proteins migrate through the gel faster than bigger proteins. The proteins are then transferred onto a membrane of nitrocellulose or polyvinylidene fluoride (PVDF). The detection of protein on the membrane is based on the use of a primary antibody binding specifically to the protein of interest, and a secondary enzyme-conjugated antibody. A substrate is added, producing adetectable product upon reaction with the enzyme. The product can be
colorimetric, chemiluminescent or fluorescent. In papers III and IV, primary mouse monoclonal antibodies against ΔNp73, TAp73, and p53 were used. An HRP-conjugated secondary antibody was used together with an enhanced chemiluminescence (ECL) assay. The chemiluminescent product was detected by a digital charge-coupled device (CCD) camera (paper III) or by photographic film (paper IV). The luminescence is produced in proportion to the amount of protein on the membrane.

**Cell viability assays**

**XTT assay**

In papers III and IV, the XTT assay was used to study cellular viability following cytotoxic treatment. The assay is based on the cleavage of the yellow tetrazolium salt XTT (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carbox-anilide) into a soluble orange formazan dye. This reaction is attributed mainly to the succinate-tetrazolium reductase system in the mitochondria of metabolically active cells. Briefly, the colon cancer cells were seeded in microplates and treated with cisplatin or oxaliplatin. After 48 and 72 hours of treatment, the XTT reagent was added. The amount of orange formazan dye produced was measured spectrophotometrically at 450 nm, and is proportional to the number of viable cells.

**Colony forming assay**

The colony-forming assay is a method of studying the effect of various agents, such as cytotoxic drugs, on the survival and proliferation of cells. Cells are seeded as single cells and then left to grow. After a period of time, a certain proportion of these cells will have formed colonies. If the cells are treated with a DNA-damaging agent, the number of proliferating cells may decrease, and fewer colonies are formed. By comparing the number of colonies formed by treated cells with the number of colonies formed by control cells, a survival fraction can be calculated. This fraction is a measurement of the effect of the studied agent on the viability of the cells. In paper III, HCT116 and HT29 colon cancer cells were seeded as single cells with or without cisplatin. The cells were grown for 7 days, after which the colonies were fixed and stained. The number of colonies visible to the naked eye was counted and a survival fraction between cisplatin treated and control cells was calculated.
Apoptosis detection

M30
In paper III, apoptosis was quantitatively detected using the M30-Apoptosense ELISA kit (Peviva, Bromma, Sweden) according to the manufacturer’s instructions. The assay is based on the M30 monoclonal antibody. M30 is a neo-epitope that is exposed in epithelial cells as the epithelial cell-specific marker cytokeratin 18 (CK18) is cleaved by caspases during early apoptosis. The M30 epitope was characterized in 1999 by Leers et al, who also characterized the antibody for detecting the epitope. The M30 Apoptosense ELISA is a solid-phase sandwich enzyme immunoassay that measures the levels of soluble caspase-cleaved CK18 containing the M30 neo-epitope. Control, standards and samples react with the solid capture antibody M5 directed against CK18 and the HRP-conjugated M30 antibody directed against the M30 neo-epitope. Unbound conjugate is removed and an HRP substrate is added, colour develops and absorbance is read. The colour development is directly proportional to the concentration of M30 in the samples.

DAPI
This method of studying the apoptotic process is based on the morphological changes occurring in apoptotic cells. The cells are stained with the dye diamidino-2-phenylindole (DAPI), which passes living cell membranes and binds to the DNA. DAPI is a blue fluorescent that is visible under UV-light, allowing the cell nuclei to be studied under a fluorescence microscope. Apoptotic cells can be distinguished from non-apoptotic cells by morphological hallmarks. While normal cell nuclei are round, even and uniformly stained, apoptotic cell nuclei display irregular edges, chromosome condensation, denser colouring and apoptotic bodies. In paper III, DAPI was used to confirm the results of the M30 apoptotic assay.

miRNA qPCR assays
The TaqMan® MicroRNA assay is a pre-formulated primer and probe set for detection of mature miRNAs using real-time PCR instruments. In paper IV, the expression levels of 377 microRNAs and 7 control assays were analysed using the TaqMan® MicroRNA Array Set v2.0, Card A (Applied Biosystems, Foster City, CA). The miRNAs on card A contains
primarily miRNAs that are functionally defined, and are commonly and/or highly expressed.

**RNA extraction and reverse transcription**

48 hours after treatment with oxaliplatin total RNA was extracted using the mirVana™ miRNA Isolation Kit (Invitrogen). Briefly, the samples were lysed in a denaturing lysis solution stabilizing RNA and inactivating RNases. The lysate was then extracted once with Acid-Phenol:Chloroform removing most of the other cellular components. Ethanol was then added to the samples, which were further purified over a glass-fibre filter. After washing, the RNA was eluted with a low ionic-strength solution.

Since a PCR reaction can only use DNA as a template, the isolated RNA was reversely transcribed into complementary DNA (cDNA) using the TaqMan® MicroRNA reverse transcription kit and Megaplex™ RT primers, human pool A v2.0 (Applied Biosystems). The cDNA samples were pre-amplified using Megaplex™ PreAmp primers and TaqMan® Preamp master mix (Applied Biosystems).

**miRNA qPCR arrays and data analysis**

The expression levels of 377 microRNAs and 7 control assays were analysed using the TaqMan® MicroRNA Array Set v2.0, Card A (Applied Biosystems). In this step, the cDNA from the reverse transcription reaction was amplified in a 40 cycle quantitative PCR (qPCR). A qPCR is based on a conventional PCR, copying and amplifying the DNA. In a qPCR, the amplification is monitored in real time using fluorescent probes. The probes are sequence-specific oligonucleotides that are labelled with two different fluorescent molecules: the reporter and the quencher. The quencher absorbs the fluorescence from the reporter as long as the probe is intact, but when the template DNA is amplified the probe is broken apart, separating the reporter and the quencher. The qPCR instrument can then register the fluorescent signal from the reporter. In our study, the 7900HT Fast Real-Time PCR System (Applied Biosystems) was used. The SDS software (Applied Biosystems) was used to calculate at which cycle the fluorescence reaches a certain threshold level (C_t). The higher the concentration of the target sequence in a sample, the lower the C_t-value will be. Theoretically, the PCR doubles the product with each cycle, meaning that a difference of 1 C_t between two samples corresponds to a double amount of target sequence in the sample with the lower C_t.
Statistical analysis

The $\chi^2$ test was used in paper I to determine differences in PINCH expression among normal mucosa, primary and metastatic colorectal cancer, as well as relationship of PINCH expression in primary colorectal cancer with clinicopathological variables. The $\chi^2$-test was also used in paper II in order to determine the relationship of the p73 genotype, regarding the G4C14→A4T14 polymorphism, with different variables.

Cox's proportional hazard model was used in papers I and II to test relationship between PINCH expression/p73 genotype and patients’ survival. The Kaplan-Meier method was used to calculate survival curves. In paper II, the AT/AT genotype was grouped together with the GC/AT genotype in the statistical analysis because of the low number of patients.

In paper III, all experiments were performed in triplicates on three different occasions. Student’s t-test was performed to evaluate statistical significance. The results were presented as means±standard error of mean (SEM).

In paper I-III, all p-values were two-sided, and p-values of less than 0.05 were considered statistically significant.

In paper IV, the $C_t$-values were normalised with qPCRNorm quantile normalisation, a data-driven normalisation strategy for high-throughput qPCR data [92]. The comparative threshold cycle method was used to calculate the amplification factor. Drug-induced modulation of miRNA gene expression was calculated using the $\Delta\Delta C_t$ method, using the untreated control as calibrator sample. N-fold change of the miRNA expression between oxaliplatin treated and untreated samples was obtained using the formula $2^{-\Delta\Delta C_t}$. The results were presented as N-fold change.
Results and Discussion

Paper I

In this study we investigated the relationship of PINCH protein expression with survival and clinicopathological variables in colorectal cancer, and the effect of PINCH expression on outcome of adjuvant chemotherapy. We found that PINCH immunohistochemical staining was present in the cytoplasm of stromal fibroblasts, whereas normal epithelial and tumour cells, except for two cases, did not show any staining. When comparing PINCH staining in samples from distant normal mucosa, adjacent normal mucosa, primary tumour and metastases, we found that strong PINCH staining was significantly more frequent in primary tumour compared to adjacent normal mucosa (p=0.0001), and in metastasis compared to primary tumour (p=0.007).

It has previously been shown that PINCH staining at the tumour invasive margin is a prognostic marker in colorectal cancer [93], implicating PINCH as a promoter of tumour invasiveness, possibly through involvement in the tumour-stromal interaction. In this study, strong staining for PINCH in the normal adjacent mucosa was related to worse survival independently of sex, age, tumour location, differentiation and stage (p = 0.044). Although histologically the mucosa adjacent to a tumour appears normal, it may differ biologically from the mucosa of a healthy individual. It is possible that this seemingly normal tissue is already involved in signalling and interactions with the tumour. The finding that PINCH expression in the adjacent normal mucosa is related to survival implicates PINCH in the biological changes occurring in the mucosa near the tumour. Furthermore, we found that the intensity of PINCH staining in poorly differentiated tumours was related to survival, with strong staining at the tumour invasive margin being significantly related to worse survival independently of sex, age and stage (p=0.013). In better differentiated tumours no such relationship was seen (p=0.40), suggesting that the impact of PINCH expression on survival is limited in well differentiated tumours.

The interaction between tumour and stroma has been recognized as an important factor influencing tumour growth and progression [94, 95]. PINCH is involved in several signalling pathways important to the tumour-stromal interaction by functioning as an adaptor protein in the integrin- and growth factor signalling pathways [67, 72]. Loss of PINCH
in *c. elegans* results in a phenotype identical to integrin-null mutants [96], indicating that PINCH is required for integrin signalling. Integrin-mediated adhesion to the ECM regulates intracellular pathways important in cell attachment, migration, proliferation [97, 98], survival and apoptosis [99]. PINCH directly associates with the proteins integrin-linked kinase (ILK) and Nck-2, forming a multi-protein complex. This prevents the proteolytic degradation of the proteins that are part of the complex [72]. Possibly, the aggressive behaviour of tumours with a high stromal expression of PINCH can be explained by an up-regulation of the signalling pathways that are dependent on the adaptor function of PINCH.

The fibroblasts of the tumour-associated stroma can affect tumour development by secreting soluble factors such as vascular endothelial growth factor (VEGF) [100] and matrix metalloproteinases (MMP:s) [101]. ILK increases the expression of VEGF, thereby stimulating angiogenesis [102]. Angiogenesis is a prerequisite for tumour growth and progression [103], and the levels of angiogenic factors such as VEGF are related to prognosis in several types of cancer, including colorectal cancer [104]. ILK overexpression also stimulates the expression of matrix metalloproteinase -9 (MMP-9) [105]. Metalloproteinases are zinc-dependent endopeptidases that degrade components of the extracellular matrix (ECM) [106], a process that is necessary for angiogenesis, tumour invasion and metastasis to occur [107]. In particular, MMP-9 plays a key role in the degradation of several components of the ECM [108], and has been found to be over-expressed in several types of cancer and to be associated with a worse prognosis [109, 110].

Remodelling of the ECM consists of breakdown and neo-synthesis of ECM components [111], and is a necessary process in tumour growth and progression [112]. Assembly of the ECM component fibronectin is regulated by ILK in a process requiring PINCH [71]. Therefore, an increase in PINCH expression could be associated with enhanced assembly of fibronectin matrix. Since the fibronectin matrix has a major impact on cell adhesion, migration, and cell growth [113, 114], an increased assembly could stimulate the migratory and invasive capacity of the tumour cells. A high expression of fibronectin in tumour stroma has been correlated to lymph node metastasis, proliferation and worse survival [115]. The interaction between fibronectin and tumour cells activates various signalling pathways involved in tumour progression, leading for
example to the increased expression of metalloproteinases MMP-2 and -9 [116, 117].

PINCH forms a complex with ILK and the adaptor protein Nck-2 [67], providing a connection between the growth factor receptor-signalling pathways and the integrin-mediated pathways [66]. Nck-2 recognizes several key components of growth factor receptor kinase-signalling pathways, including EGF receptors, PDGF receptor-β and insulin receptor substrate-1 (IRS-1) [67]. Growth factors are important regulators of the tumour-stromal interaction, and for some carcinomas an increase in growth factor receptors in stromal cells is thought to be an essential part in the tumour-stromal signalling [118, 119].

Since PINCH expression was related to prognosis, we further investigated PINCH in relation to treatment response. In our group of patients, 27 patients received adjuvant chemotherapy. We found that patients with weak stromal staining for PINCH in the entire primary tumour (p = 0.010), inner tumour area (p = 0.013) or at the invasive margin (p = 0.013) receiving adjuvant chemotherapy had significantly better survival than those without chemotherapy. In patients with strong stromal staining for PINCH in the entire tumour (p = 0.13), inner tumour area (p = 0.16) or at the invasive margin (p = 0.16) chemotherapy was not significantly related to survival. This indicates that PINCH could be one factor influencing the outcome of adjuvant chemotherapy. However, in the multivariate analysis the relationship between PINCH staining and chemotherapy outcome was not significant. Possibly, this could be due to the relationship of PINCH expression with inflammatory infiltration and differentiation grade, known prognostic factors in colorectal cancer [120, 121]. Further, the relatively low number of patients may contribute to the lack of significant results.
Paper II

The G4C14→A4T14 polymorphism is located just upstream of the initiating codon in exon 2 of the p73 gene. It does not cause an amino acid shift, but is located in a region that might theoretically form a stem loop structure. It is therefore speculated that the polymorphism might affect translational efficiency or splicing of the transcript [38]. In this study we investigated if the G4C14→A4T14 polymorphism was related to survival in 138 rectal cancer patients who did or did not receive preoperative radiotherapy. Further, we studied whether the polymorphism was related to clinicopathological variables or expression of other proteins. In the overall group of patients, we found that the polymorphism was not related to any of the clinicopathological variables, nor to disease-free survival (p=0.54). However, in the radiotherapy group, patients with the GC/GC genotype slightly tended to have better survival, although the difference was not significant (p = 0.20).

The role of the p73 G4C14→A4T14 polymorphism in relation to cancer risk has been investigated in populations with different genetic and social background, but the results are inconsistent. Studies have shown that the AT/AT genotype or the AT allele is associated with a decreased risk of some cancer types [122, 123], while other studies have shown the opposite [124-127]. In colorectal cancer, it has been shown the AT/AT genotype increases the risk [128], however, the GC/GC genotype is associated with worse survival. Overexpression of p73 has been connected to worse prognosis in colorectal cancer [62], and it is speculated that the polymorphism alters p73 protein expression [128]. However, previous immunohistochemical studies of p73 on the material used in this study [85] revealed no significant relationship between genotype and staining intensity. However, only total p73 expression was examined due to the lack of more specific antibodies at the time, and p73 is expressed as two main isoforms: TAp73 and ΔNp73. TAp73 is the full-length pro-apoptotic variant of the protein, homologous to p53. The N-terminally truncated form, ΔNp73, is a product of an alternative promoter located in intron 3 [129]. The polymorphism is located just upstream of the transcriptional start site [38], and it is speculated that the polymorphism may therefore alter the balance between the p73 isoforms, favouring the expression of a certain isoform. To further investigate the impact of the p73 polymorphism genotypes in response to radiotherapy it would be of interest to compare the genotypes of the polymorphism with the expression of the two different N-terminal isoforms of p73.
Further, we studied whether the polymorphism was related to expression of other proteins, and found a significant relationship in the radiotherapy group between the AT allele and the expression of p53 and survivin. Patients in the radiotherapy group carrying the AT allele more frequently had positive immunohistochemical staining for p53 (indicating p53 mutation) \( (p=0.001) \) and strong staining for the protein survivin \( (p=0.03) \) than patients with the GC/GC genotype. Considering this relationship, a comparison between patients with GC/GC genotype, negative p53 expression and weak survivin expression, with all other patients was done. In the radiotherapy group, we found that the patients with GC/GC genotype, negative p53 and weak survivin expression had significantly better survival than other genotype/phenotype combinations, independently of TNM stage and tumour differentiation \( (p=0.01) \). There was no survival significance for the three proteins in the non-radiotherapy group \( (p = 0.37) \). The combination of negative p53 and weak survivin expression alone was not significantly related to better disease-free survival, although previous studies have shown p53 IHC staining to be a predictive factor for preoperative radiotherapy [47]. In our study, it seems that the combination of p73, p53 and survivin or their interactions may play a role in predicting prognosis in rectal cancer patients with radiotherapy.

A possible relationship between p53, p73 and survivin has been seen in several studies [130-132]. Survivin is an anti-apoptotic protein that regulates the cell cycle [133] and inhibits apoptosis [134]. Survivin is undetectable or only weakly expressed in most normal adult tissues, but has been shown to be expressed in several types of tumours, including colorectal cancer [135]. Survivin increases radioresistance and high survivin expression is associated with a worse outcome in rectal cancer patients that have had pre-operative radiotherapy [136, 137]. Wild-type p53 seems to repress survivin, either transcriptionally via E2F1 or via acetylation of the promoter region of survivin, containing two p53 binding elements [130, 132]. Also, ectopic survivin affects the levels of p53 in breast cancer cell lines, possibly through a survivin-dependent inhibition of caspases, in turn affecting mdm2 and the ubiquination of p53. Further, forced expression of survivin increased the levels of both TAp73 and ΔNp73 [131]. It has also been suggested that TAp73 is capable of down-regulating survivin [138].
To summarise our study, the p73 G4C14→A4T14 polymorphism might be a factor influencing the response to preoperative radiotherapy, specifically in combination with wild type p53 and low levels of survivin.
Paper III

In this in-vitro study, we investigated the effect of the p73 isoform ΔNp73β on the cytotoxicity of cisplatin in the colon cancer cell lines HCT116p53+/+, HCT116p53−/− and HT29 by over-expressing ΔNp73β. Protein expression of ΔNp73β markedly increased in all cell lines after transfection. However, in both HCT116 cell lines, ΔNp73β protein levels decreased in a dose-dependent manner in response to cisplatin. Protein expression of the p73 isoform TAp73 increased after cisplatin treatment in both ΔNp73β- and mock-transfected cells. In the HCT116p53+/+ cells p53 expression increased in response to cisplatin, and also in ΔNp73β-transfected cells, even without cisplatin treatment.

It has been shown in the sarcoma cell line SAOS-2 that ΔNp73 is rapidly degraded in a proteasome-dependent manner in response to DNA-damage [139]. The degradation of ΔNp73 may be a safety mechanism, allowing cell cycle arrest and apoptosis to occur properly in damaged cells without inhibition from the anti-apoptotic ΔNp73. In contrast, cisplatin up-regulates ΔNp73 in SH-SY5Y neuroblastoma cells [140, 141]. In our study, ΔNp73β was degraded in a dose dependent manner in response to cisplatin, so any resistance to cisplatin treatment conferred by ΔNp73β should be minor at the concentrations used in the cellular viability and apoptosis assays. However, a relatively low concentration of cisplatin was used in the colony-forming assay, and the survival fractions slightly increased in ΔNp73β-transfected cells compared to mock-transfected cells, indicating resistance to cisplatin treatment. The difference was not, however, significant.

In this study, p53 expression increased in response to cisplatin, and also in ΔNp73β-transfected cells, even without cisplatin treatment. p53 contributes to treatment response by inducing cell cycle arrest and apoptosis [90], and we found that HCT116p53−/− cells were less sensitive to cisplatin treatment than HCT116p53+/+ cells, confirming the involvement of p53. The increase in p53 protein in cells overexpressing ΔNp73β could be due to blocking of the mdm2 promoter as ΔNp73β has previously been shown to reduce the transcriptional activation of mdm2 [42, 142]. Mdm2 promotes the ubiquitin-mediated degradation of p53 [42], and high levels of ΔNp73β may therefore result in reduced degradation of p53.

Expression of TAp73 protein was also increased after cisplatin treatment. Inhibition of TAp73 has been shown to increase resistance to cisplatin treatment [52], suggesting that TAp73 is involved in cisplatin-induced cell cycle arrest and apoptosis. It has also been reported that p73
is not induced in response to cisplatin in the HCT116 cell line as a result of deficient mismatch repair [143]. Cisplatin increases the half-life of p73 by activating the tyrosine kinase c-abl in mismatch-repair-proficient cells [143]. However, p73 has been stabilized by the transcription factor c-Jun in response to cisplatin, in cells defective in c-abl binding and phosphorylation [144], suggesting alternative pathways for p73 induction independent of c-abl and the mismatch-repair signalling pathway.

When studying cellular viability, we found that cisplatin significantly decreased viability in all three cell lines. In HCT116p53+/+ cells treatment with 20 µM of cisplatin for 48 and 72 hours decreased viability in ΔNp73β-transfected (p=0.026 and 0.008) and mock-transfected cells (p=0.023 and 0.015). Similar results were seen in the HCT116p53-/- cells after 48 and 72 hours of treatment both in ΔNp73β-transfected cells (p=0.048 and 0.09) and mock-transfected cells (p=0.005 and 0.028). In HT29 cells, cellular viability was decreased after 72 hours of treatment both in ΔNp73β-transfected cells (p=0.001) and mock-transfected cells (p=0.045). Regarding the effect of ΔNp73β over-expression, ΔNp73β-transfected cells were significantly more viable than mock-transfected cells at 48 hours after transfection (p=0.005 and 0.01 for HCT116p53+/+ and HCT116p53-/-, respectively). In HCT116p53+/+ and HT29 cells treated with 20 µM of cisplatin, cellular viability of ΔNp73β-transfected cells was significantly higher than mock-transfected cells (p=0.006 and 0.001). Similar results, although not significant, were seen in HCT116p53-/- cells.

Apoptosis was evaluated in the HCT116 cell lines, and we found that apoptotic cells significantly increased after treatment with 20 µM of cisplatin in HCT116p53+/+ cells (p=0.049 and 0.004 for ΔNp73β- and mock-transfected cells, respectively). In HCT116p53-/- cells apoptosis tended to increase after cisplatin treatment, although not significantly. No difference between ΔNp73β- and mock-transfected cells were seen in either of the cell lines. The lack of significant differences between ΔNp73β- and mock-transfected cells regarding apoptosis indicates that the differences in cellular viability are not primarily due to increased apoptosis. A reduction in cell cycle arrest could explain why ΔNp73β-transfected cells were more viable.

Although we could not find that ΔNp73β had any effect on apoptosis, other studies have shown that overexpression of ΔNp73 protects cells from apoptosis by inhibiting p53 target genes such as p21 [139], while down-regulation of ΔNp73 increases apoptosis [145]. Up-regulation of ΔNp73 in tumours may disturb apoptotic signalling.
pathways by interfering with p53 and TAp73, and high expression of ΔNp73 has been correlated with worse patient outcome [146, 147] and resistance to DNA-damaging drugs [148, 149]. However, the effect of the ΔNp73β isoform remains indistinct. Unlike ΔNp73α, ΔNp73β has been shown to possess the capability of activating some p53-responsive genes, thereby inducing both cell cycle arrest and apoptosis [150]. Resulting from the use of the alternative intron 3 promoter, the ΔNp73 isoforms contain 13 unique residues at the N-terminus shown to contain an activation domain [150]. The ΔNp73α and -β isoforms are identical apart from the C-terminal where the β isoform lacks exon 13 [38], suggesting that the C-terminal of the α isoform represses transcription. Both ΔNp73α and -β have been shown to inhibit the transcription of p53 responsive genes, although the α isoform is much more potent than the β isoform, which shows some intrinsic transactivation capacity [151]. Also for TAp73, the α isoform has lower transactivation ability than the β isoform [152].

In this study, we have demonstrated that cisplatin decreases clonogenic potential and cellular viability while increasing apoptosis. Overexpression of ΔNp73β increased cellular viability in HT29 cells as well as in HCT116p53+/+ and HCT116p53−/− cells.
Paper IV

In this study, we investigated whether exposure to oxaliplatin alters the expression of miRNAs in colon cancer cells, and whether the miRNA expression profiles after oxaliplatin treatment are influenced by p53 and p73. siRNA was used to knock down the expression of the full-length, pro-apoptotic TAp73 in HCT116\(^{p53+}\) and HCT116\(^{p53-}\) cells. 72 hours after transfection, protein expression became markedly reduced as determined by western blot. Cellular viability decreased in a dose-dependent manner in response to oxaliplatin, with p53 wild-type cells being more sensitive than p53 negative cells.

Gene expression levels of 377 miRNAs and 7 control assays were analysed after oxaliplatin treatment. A number of miRNAs were affected by the oxaliplatin treatment, and the results differed depending on p53 and p73 status. The majority of the miRNAs that were dysregulated by oxaliplatin has previously been associated with cancer disease. Only those miRNAs with at least two-fold change between control and oxaliplatin treated cells were considered. In the HCT116\(^{p53+}\) cells 16 miRNAs were up-regulated after exposure to oxaliplatin and 15 miRNAs were up-regulated in the HCT116\(^{p53-}\) cells (Table 2). 11 of the up-regulated miRNAs were common to both cell lines. Of interest is that several of the up-regulated miRNAs have been reported to be down-regulated or to have tumour suppressive properties in different cancer types, including miR-542-3p [153], miR-511 [154] and miR-98 [155]. Some miRNAs have also been related to chemotherapeutic response or chemosensitivity, such as miR-519d [156], miR-142-5p [157] and miR-143. miR-143 is down-regulated and a putative tumour suppressor in colorectal cancer [158]. Further, it has been shown that miR-143 increases chemosensitivity to oxaliplatin in colorectal cancer cells [159].

Some miRNAs in this study were found to be dysregulated in response to oxaliplatin exclusively in the HCT116\(^{p53+}\) or the HCT116\(^{p53-}\) cell line, indicative of the role p53 plays in the response to chemotherapy as well as in the regulation of miRNAs. p53 is an important molecular effector of oxaliplatin activity, and p53-mutant cells are more resistant to oxaliplatin treatment than p53 wild-type cells [160]. p53 is a transcription factor that regulates the expression of genes related to cell cycle arrest, apoptosis and DNA repair [161-163], as well as a considerable number of miRNAs [164]. In our study, miRNAs exclusively up-regulated in the HCT116\(^{p53+}\) cell line included miR-34c, miR-132, miR-518f, miR-758, and miR-521, and in the HCT116\(^{p53-}\) cell line miR-18b, miR-204, miR-127 and miR-
Several of these miRNAs, including miR-34c [165], miR-132 [166], miR-18b [167], miR-204 [168, 169] and miR-127 [170, 171] have been reported to be involved in chemoresponse. Especially, the miR-34-family is known to be directly regulated by p53 and is up-regulated in response to DNA-damage in p53 wild-type cells, where it exerts anti-proliferative effects [172]. Further, MiR-34c has been found to be down-regulated in colorectal cancer [173]. miRNAs exclusively down-regulated in our study included miR-570, miR-331-5p, miR-503 and miR-548a in HCT116p53+/+ cells, and miR-205 and miR-153 in HCT116p53-/- cells. The p53-family regulates the processing of several miRNAs, including miR-503 [174], miR-503 [175], miR-331-5p [176] and miR-153 [177] have been shown to be involved in chemoresistance. miR-153 in particular, is up-regulated in colorectal cancer where it increases invasiveness and resistance to cisplatin and oxaliplatin [177].

Table 1: miRNAs up-regulated in colon cancer cell lines HCT116p53+/+ and HCT116p53-/- after exposure to oxaliplatin (2µM).

<table>
<thead>
<tr>
<th>miRNA</th>
<th>HCT116p53+/+ Fold change</th>
<th>HCT116p53-/- Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-34c</td>
<td>48.52356615</td>
<td>91.12782036</td>
</tr>
<tr>
<td>hsa-miR-519d</td>
<td>46.74359372</td>
<td>20.67975469</td>
</tr>
<tr>
<td>hsa-miR-615</td>
<td>15.7519451</td>
<td>13.32361577</td>
</tr>
<tr>
<td>hsa-miR-518b</td>
<td>8.900425141</td>
<td>12.40290647</td>
</tr>
<tr>
<td>hsa-miR-142-5p</td>
<td>8.336024903</td>
<td>7.724223094</td>
</tr>
<tr>
<td>hsa-miR-521a</td>
<td>7.982827924</td>
<td>6.68393781</td>
</tr>
<tr>
<td>hsa-miR-518f</td>
<td>6.389308986</td>
<td>5.8568485</td>
</tr>
<tr>
<td>hsa-miR-758a</td>
<td>4.62524895</td>
<td>5.488574873</td>
</tr>
<tr>
<td>hsa-miR-618</td>
<td>4.207956381</td>
<td>4.827132463</td>
</tr>
<tr>
<td>hsa-miR-143</td>
<td>3.533906411</td>
<td>4.796016763</td>
</tr>
<tr>
<td>hsa-miR-133a</td>
<td>3.242127888</td>
<td>4.296335446</td>
</tr>
<tr>
<td>hsa-miR-511</td>
<td>2.831831648</td>
<td>4.011459782</td>
</tr>
<tr>
<td>hsa-miR-545</td>
<td>2.393026756</td>
<td>3.915706949</td>
</tr>
<tr>
<td>hsa-miR-132a</td>
<td>2.212488858</td>
<td>2.504146124</td>
</tr>
<tr>
<td>hsa-miR-98</td>
<td>2.172602385</td>
<td>2.020914566</td>
</tr>
<tr>
<td>hsa-miR-323-3p</td>
<td>2.136596898</td>
<td></td>
</tr>
</tbody>
</table>

* Up-regulated exclusively in HCT116p53+/+

* Up-regulated exclusively in HCT116p53-/-
Among the miRNAs that were up-regulated in response to oxaliplatin in this study, the consistent trend is that they are commonly down-regulated in cancer, play tumour suppressive roles and increase chemosensitivity. However, among the miRNAs that were down-regulated (Table 2) after oxaliplatin treatment, the opposite pattern was not found. Some of the down-regulated miRNAs are considered to act as tumour suppressors, for example miR-125a-3p [178], miR-433 [179] and miR-379 [180], and several have shown conflicting results in various studies. Only a few display oncogenic properties or chemoresistance, including miR-367 [181, 182] and miR-215 [183, 184].

p53 is well known to regulate the expression of a wide range of miRNAs, but little is known about p73 in this context. p73, like p53, is involved in the response to several chemotherapeutic drugs [52], including oxaliplatin [160]. In this study, the full-length pro-apoptotic isoform TAp73 was knocked down using siRNA, in order to determine whether TAp73 could influence the expression of miRNAs in a similar way as p53. While the majority of miRNAs that were dysregulated by oxaliplatin were still dysregulated after TAp73 knock-down, there were however some exceptions. These included miR-519d, miR-545, miR-618 and miR-98, miR-132, miR-518f, miR-758, miR-127 and miR-654 which were up-regulated in the HCT116p53+/+ and/or HCT116p53-/- but not when TAp73 was knocked down. Among the down-regulated miRNAs, miR-433 and miR-379 (down-regulated in both cell lines), and miR-205 (down-regulated in HCT116p53-/- cells), were not found to be down-regulated after TAp73 knockdown. miR-205 has previously been reported to be a target of p73 [185], and we found that the expression level of miR-205 was undetectable in both cell lines with and without oxaliplatin treatment when TAp73 gene expression was knocked down.

To conclude, we found that oxaliplatin modulates the miRNA gene expression profile. This could indicate that some of the pharmacodynamic mechanisms of oxaliplatin in colon cancer are due to the effect on miRNA expression, particularly as many of the dysregulated miRNAs are related to cancer and/or chemoresponse. Both p53 and TAp73 was found to modulate the miRNA expression profiles after oxaliplatin treatment, possibly indicating that p53 and TAp73 could regulate the role of miRNAs in chemosensitivity.
**Table 2:** miRNAs down-regulated in colon cancer cell lines HCT116<sup>p53+/+</sup> and HCT116<sup>p53-/−</sup> after exposure to oxaliplatin (2µM).

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Fold change</th>
<th>miRNA</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-367</td>
<td>-101,5883995</td>
<td>hsa-miR-153</td>
<td>-30,51328813</td>
</tr>
<tr>
<td>hsa-miR-125a-3p</td>
<td>-38,94856631</td>
<td>hsa-miR-146b-3p</td>
<td>-6,14762181</td>
</tr>
<tr>
<td>hsa-miR-146b-3p</td>
<td>-36,57468827</td>
<td>hsa-miR-215</td>
<td>-6,002523569</td>
</tr>
<tr>
<td>hsa-miR-199b</td>
<td>-17,19593194</td>
<td>hsa-miR-433</td>
<td>-5,849220513</td>
</tr>
<tr>
<td>hsa-miR-548a</td>
<td>-15,61460848</td>
<td>hsa-miR-199b</td>
<td>-4,949409242</td>
</tr>
<tr>
<td>hsa-miR-433</td>
<td>-8,900425106</td>
<td>hsa-miR-379</td>
<td>-3,226839904</td>
</tr>
<tr>
<td>hsa-miR-379</td>
<td>-6,865120645</td>
<td>hsa-miR-576-3p</td>
<td>-3,223931361</td>
</tr>
<tr>
<td>hsa-miR-302a</td>
<td>-5,154826954</td>
<td>hsa-miR-205</td>
<td>-3,223931361</td>
</tr>
<tr>
<td>hsa-miR-570</td>
<td>-4,904072444</td>
<td>hsa-miR-125a-3p</td>
<td>-3,09086122</td>
</tr>
<tr>
<td>hsa-miR-503</td>
<td>-4,523923221</td>
<td>hsa-miR-494</td>
<td>-3,053183747</td>
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<tr>
<td>hsa-miR-331-5p</td>
<td>-4,508077562</td>
<td>hsa-miR-367</td>
<td>-2,832397343</td>
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<tr>
<td>hsa-miR-215</td>
<td>-3,632733479</td>
<td>hsa-miR-302a</td>
<td>-2,58300795</td>
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<tr>
<td>hsa-miR-494</td>
<td>-3,015915687</td>
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</tr>
<tr>
<td>hsa-miR-576-3p</td>
<td>-2,059959658</td>
<td></td>
<td></td>
</tr>
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</table>

<sup>a</sup> Down-regulated exclusively in HCT116<sup>p53+/+</sup>

<sup>b</sup> Down-regulated exclusively in HCT116<sup>p53-/−</sup>
Conclusions

Based on the results of paper I-IV, the conclusions of this thesis are:

- Stromal immunohistochemical staining for PINCH in adjacent normal mucosa was related to survival in colorectal cancer patients. Further, PINCH staining at the tumour invasive margin was related to survival in poorly differentiated tumours but not in better differentiated tumours. Adjuvant chemotherapy was related to survival in patients with weak stromal staining for PINCH, but not in patients with strong staining. Taken together, our results indicate that PINCH could be one factor influencing the prognosis in colorectal cancer patients.

- In rectal cancer patients treated with radiotherapy, the patients with the GC/GC genotype tended to survive longer, indicating that the p73 G4C14→A4T14 polymorphism might be a factor influencing the response to preoperative radiotherapy. The effect was significant in combination with wild type p53 and low levels of survivin.

- Cisplatin decreased clonogenic potential and cellular viability, increased apoptosis and up-regulated p53 and TAp73 in HCT116 cells. Overexpression of ΔNp73β increased cellular viability in HT29 and HCT116 cells. Resistance to cisplatin treatment increased slightly in ΔNp73β-overexpressing HCT116 cells.

- Oxaliplatin modulated the miRNA gene expression profile in HCT116 cells, and many of the dysregulated miRNAs are related to cancer and/or chemoresponse. The effect of oxaliplatin on the miRNA expression profile differed depending on p53 and TAp73 status.
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My near and dear ones: Mum, dad, sisters, brother and friends, the best ones you could ever have!

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