DEPARTMENT OF CLINICAL SCIENCE AND EDUCATION,
SÖDERSJUKHUSET
Karolinska Institutet, Stockholm, Sweden

THE CLINICAL USE OF GENETIC ANALYSES IN COLORECTAL CANCER

Susanne Tumlin Ekelund

Karolinska Institutet

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To Robert, Sofia, Klara and Pongo

ABSTRACT

Background: Colorectal cancer (CRC) is a common global disease, with a mortality rate of almost 50%. Prognosis is mainly based on the TNM classification. Surgical interventions have the potential of being curative in patients with stage I-III CRC. Adjuvant treatment with chemotherapy enhances the survival rate, especially in stage III cancer. Chemotherapy does, however, have significant side effects. Therefore, refinement of therapies based on improved prognostic ability on an individual level is essential. One way to achieve this could be to examine the tumours on a molecular level and not just histologically. Recent studies have shown that K-ras mutation is a negative predictor to anti-EGFR (epidermal growth factor receptor) therapy. MicroRNAs were discovered only 2 decades ago but are now the most promising biomarkers in many cancers. In this thesis, the first two papers focus on genetic changes in the tumour and in lymph nodes, in particular looking at the oncogene K-ras. The last two papers focus on microRNAs in tumours and in blood serum. In studies 2, 3 and 4, our findings on the correlation of some molecular changes to prognosis is described.

Study I:

17 tumours from CRC patients were divided in to small cubes. DNA was extracted from each biopsy and the occurrence of K-ras mutations, methylation of p16 and MGMT, and loss of heterozygosity (LOH) at 5q, 17p and 18q were analysed.

We found that the distribution of methylated p16 and MGMT and LOH at 5q, 17 p and 18q are heterogeneous and present in a large majority of CRC tumours, thereby of limited prognostic value. However, K-ras mutation appears more homogeneously spread, a finding of clinical relevance for the use of biopsies to predict anti-EGFR response.

Study II:

99 stage II CRC patients with histologically normal lymph nodes were included. DNA was extracted from lymph nodes, tumours and normal mucosa and the K-ras status was analysed and correlated to prognosis.

Of the tumours, 34/99 were identified as positive for K-ras mutations. Of these, 10 patients also expressed K-ras mutations in their lymph nodes. Of the 10 patients with positive lymph nodes, 7 (70%) relapsed and died from the disease within 60 months compared to 8/ 24 (33%) with K-ras negative lymph nodes.

Study III:

50 CRC patients were studied. RNA was extracted from the tumours. 5 patients with short and 5 patients with long survival were selected for SYBR-green quantitative PCR-based array to screen for differently expressed microRNAs. From this screening, 6 candidate prognostic microRNAs were validated using TaqMan quantitative PCR in all 50 patients.

We found that high expression of *miR-185* and low expression of *miR-133b* correlated to poor survival (p=0.001 and p=0.028, respectively) and metastasis (p=0.007 and p=0.036, respectively) in CRC.

Study IV:
16 CRC patients and one with a large adenoma in the colon were included. All patients underwent radical (R0) surgery. Blood serum was collected prior to and 30 days after surgery. 3 microRNAs were analysed with Taqman qPCR (miR-21, miR-133b and miR-185).
The serum levels of mir-21 were not affected by radical tumour resection. There was a significant decrease in the level of miR-133b among the patients following surgery, and an overall reduction of miR-185. There was no correlation between intra-individual changes in serum levels pre- and postoperatively to disease outcome, or between baseline levels and the risk of recurrent disease.

LIST OF PUBLICATIONS

I. Tissue sampling for mutation analysis in colorectal cancer: K-ras is homogeneously distributed throughout the tumor tissue

Susanne Ekelund, Nikos Papadogiannakis, Hans Olivecrona, Ulrik Lindforss

Oncology Reports, January 2011, pages 253-258

II. The prognostic significance of K-ras mutations in regional lymph nodes after radical resection for stage II colorectal carcinoma

Susanne Tumlin-Ekelund, Nikos Papadogiannakis, Kjell Gullberg, Leif Törkvist, Greger Lindberg, Achilleas Karkamanis, Hans Olivecrona, Ulrik Lindforss

Submitted

III. miR-185 and miR-133b deregulation is associated with overall survival and metastasis in colorectal cancer

Pinar Akcakaya, Susanne Ekelund, Iryna Kolosenko, Stefano Caramuta, Deniz M. Özata, Hong Xie, Ulrik Lindforss, Hans Olivecrona, Weng-Onn Lui

International Journal of Oncology, August 2011, pages 311-318

IV. The Effect of Radical Tumor Resection on Serum MicroRNA in Cancer Patients – a Long-term Follow-up of Patients with Colorectal Carcinoma

Susanne Tumlin Ekelund, Ulrik Lindforss, Mika Leinonen, Hans Olivecrona

Manuscript

CONTENTS

LIST OF ABBREVIATIONS

APC	Adenomatous Polyposis Coli
CEA	Carcinoembryonic antigen
CIMP	CpG Island Methylator Phenotype
CIN	Chromosomal instability
DNA	Deoxyribonucleic acid
EGFR	Epidermal derived growth factor
HNPCC	Hereditary non polyposis colorectal cancer
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
K-ras	Kirsten rat sarcoma viral oncogene homolog
LOH	Loss of heterozygosity
miR/miRNA	microRNA
MMR gene	Mismatch repair gene
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MSI	Microsatellite instability
MSS	Microsatellite stable
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
RNA	Ribonucleic acid
TGF	Tumour growth factor
TGGE	Temperature gradient gel electrophoresis
TNM	Tumour nodule metastasis
TSG	Tumour suppressor gene
VEGF	Vascular endothelial growth factor

FOREWORD

Colorectal cancer (CRC) is a complex disease process. The basic aetiology begins within a normal epithelium cell, in the colon- or rectal mucosa, which transforms and develops into a tumour cell, eventually becoming malignant. There are varying grades of cell dysplasia depending on the stage of disease process. Despite improvements in diagnosis, surgical techniques, and chemotherapeutic regimens, CRC is still responsible for many deaths around the world. The work in this thesis focuses on studying several genetic events involved in, or consequences of, CRC. The purpose was to investigate their relationship to disease outcome, with the aim to study their usefulness in improving diagnosis and prognostic accuracy, thereby refining the possibility of applying correct treatment modalities in the individual patient.

1 Background

Epidemiology

Colorectal cancer (CRC) is the third most common cancer in men and the second most common cancer in women worldwide (1). In 2010, the Swedish National Board of Health and Welfare reported that colon cancer is the third most common cancer in Sweden irrespective of gender. The incidence for colon cancer in Sweden is approximately 4000 new cases per annum, and that of rectal cancer is approximately 2000 cases per annum. With a mortality of about 50%, 0.6 million will succumb to the disease of the estimated 1.23 million individuals affected worldwide annually. Notably the disease is more common in more developed countries than in lesser developed countries (2). The 5-year survival rates range from 90% to 10% depending on tumour progression (3). The 5-year cumulative rate for distant metastasis in Stage I, II and III (staging is described in more detail below) are 6.4, 21.4 and 48.0 per cent respectively (4). Altogether this is a common and deadly disease that requires early diagnosis and treatment to improve prognosis.

Risk factors

Most cancers are believed to develop by a combination of genetic and environmental factors. Since the disease is more common in more developed countries, many researchers have investigated environmental risk factors. It has been postulated that it takes one generation for a family that moves from one country to another to exhibit the same cancer rates as in the new environment, thereby supporting this theory of environmental modulation (5). One of the possible explanations may be due to environmental carcinogens related to industrial bi-products and synthetic products in more developed countries. Other contributing factors include a diet rich in unsaturated

fats and red meat, high total energy intake, excessive alcohol consumption and reduced physical activity, which have all been shown to enhance the risk of CRC (6, 7).

Interestingly, non steroidal anti-inflammatory drugs (NSAIDs), calcium and oestrogen appear to protect against CRC (7, 8). Patients with inflammatory bowel diseases have also been shown to be at a greater risk of developing CRC (9). The aetiology of CRC is complex and multi-factorial and the role of intestinal flora is still unclear. Intestinal flora may contribute to both increased and decreased risks of developing CRC depending on the composition. Thus several studies are being conducted to define different types of gut flora and their impact on intestinal function and subsequent disease development (10, 11).

Staging

Staging and prognosis is mainly based on the *Tumour Nodules Metastasis* (TNM) classification according to International Union Against Cancer (IUAC), and the American Joint Committee on Cancer (AJCC), as outlined in Table 1. The pathological anatomical diagnosis (PAD) is performed by a qualified pathologist under microscopic examination. To be able to stage the cancer, several histological questions need to be answered; for example, the number of lymph nodes (at least 12 lymph nodes is considered a minimal requirement), degree of differentiation of the tumour, venous and lymphatic invasion, and nerve infiltration. The TNM classification has been regularly updated and modified since it first was established. Today, the tumour infiltration evaluation is more precise and there are several subgroups in the different stages.

The basis for the utilization of adjuvant therapies, in addition to surgery, depends on the histological evaluation and the TNM classification of the tumour. Due to limitations of

using TNM as the only prognostic variable in clinical practice the consequence may be a certain degree of over-treatment of patients in advanced stages and, reciprocally, omission of a number of patients in earlier stages that may benefit from adjuvant therapy.

Table 1. Simplified TNM classification

	Tumour	Nodules	Metastasis
Stage I/ Duke´sA	T1-tumour invades submucosa T2-tumour invades muscularis propria	N0=no lymph node involvement	M0=no distant metastasis
Stage II/ Duke´sB	T3-tumour invades through m propria into the subserosa T4- tumour invades other organs or structures	N0	M0
StageIII/ Duke´sC	T1-T4	N1-2=lymph node involvement	M0
Stage IV	T1-T4	N0-N2	M1=distant metastasis present

Treatment

Three different modalities are currently used in the treatment of colorectal cancer: surgery, chemotherapy, and radiation therapy, and in many cases a combination of these. The results of surgical treatment have improved over the last few decades by implementation of the *total mesorectal excision* (TME) technique used in rectal cancer. The similar surgical approach has recently been adapted to colon cancer (12,13). The introduction of 'high ties' and higher yields of lymph node extraction in the specimen, have also improved outcome (14-16). Radiation therapy used as a target specific

treatment modality in rectal cancer, preoperatively exposing the tumour mass to highly focused high energy beams of radiation, further has improved outcome (17). Radio therapy is also used in some cases on distinct metastases. Adjuvant pharmaceutical therapies are both cytotoxic and biologic; namely: 5-FU (fluorouracil), bevacizumab, leucovorin, cetuximab, oxaliplatin and irinotecan. Over the decades, adjuvant therapies have progressively improved outcome in CRC patients. Today, some genetic mutations are also clinically important factors in the allocation of treatment modalities. The mutational status of the K-ras gene is a predictor of the outcome of anti-epidermal growth factor receptor (EGFR) treatment of metastatic CRC with the chemotherapeutic agent cetuximab (18-20).Furthermore, MSI status is a negative predictor of 5-FU treatment (21). These predictive tests are pioneering the utilization of genetic events that can tailor treatment regimen in a more precise manner based on factors beyond microscopic classification.

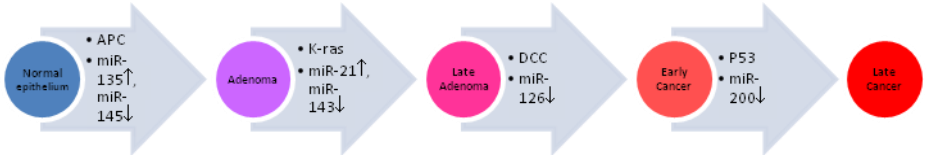
Familial cancer

Familial or inherited cancer is characterized by a germ line mutation which increases the susceptibility to acquire somatic mutations, eventually leading to cellular dysplasia and the development of cancer (22). In colorectal cancer the two most common familial disorders are the *familial adenomatous polyposis*, caused by a mutation in the APC gene, and *HNPCC* (hereditary non polyposis colon cancer), also known as Lynch disease, which is caused by a mutation in one of the mismatch repair genes (23). The work in this thesis is focused on *sporadic* cases of colorectal cancer, and excluded patients with known germ line mutations. Also in sporadic cases of the disease, mutations in the APC gene and mismatch repair genes can be seen. Thus it is important to note that genetic studies on the familial cancer cells and germ line cells have improved the understanding of tumorigenesis even in sporadic cases.

Tumorigenesis

As mentioned above, the development of colorectal cancer is a complex, heterogeneous and multi-factorial process. It may possibly start with one mutation in an epithelial cell, but, after several stepwise mutations, the neoplastic cell will eventually become malignant. Vogelstein and Fearon first described this "*Vogelgram*" pattern of tumorigenesis (24,25). This model proposes an accumulation of mutations that initially change the normal epithelium to an adenoma and then into a carcinoma, (Figure 1). The model has been modified throughout the years, and is still being updated with the discovery of new key elements in tumour development.

Figure 1. A modified "Vogelgram". Multi-step genetic mutations eventually leading to the development of cancer. Suggested deregulations of microRNAs are also shown.



The molecular defects are of two types: (i) alterations that lead to novel or increased function of oncogenes, and, (ii) alterations that lead to loss of function of tumour-suppressor genes (7). A malignant epithelial cell does not respect the epithelial membrane thus leading to eventual tumour invasion in to the surrounding structures. Furthermore, once the malignant process has begun, the cell escapes apoptosis and becomes immortalized. The abilities of the cancer cell to metastasize are induced when it acquires traits such as motility and can adapt to foreign environments, enabling it to survive in different tissue environments in the body.

Intra- and extracellular communication are essential in the normal functioning of the cell and tissue growth. The epithelial cells communicate through a multitude of different factors and receptors. When a receptor is activated in the cell membrane it starts an intracellular signalling cascade, eventually leading to the activation of transcription factors. Through the activation of these factors different cellular behaviour is accomplished, including apoptosis, migration, growth, adhesion and differentiation. In tumorigenesis, intracellular overactivation of a growth factor may result in uncontrolled proliferation, and this result from a combination of abnormal genetic and epigenetic events.

In carcinomas, extracellular communication with stromal cells, such as fibroblasts, myofibroblasts, and endothelial cells is crucial as these cells are recruited for various types of physiological support. This interdependence is manifested by the exchange of various types of mitogenic and trophic factors, for example PDGF (platelet derived growth factor), IGF (insulin-like growth factor) and VEGF (vascular endothelial growth factor) (5).

Currently, it is believed that there are three key pathways in the development of CRC: *CIN* (chromosomal instability), *MSI* (microsatellite instability), and *CIMP* (CpG island methylation phenotype). These pathways may work independent of one another, or they may overlap resulting in a multiple mutational aetiology. Details of the pathways are as discussed below.

CIN (chromosomal instability)

CIN is a type of genomic instability where changes in the chromosomal copy numbers and structure occur. In CRC, the CIN pathway leads to aneuploidy, in turn causing genetic instability and an increased rate of mutations. Further, parts of a chromosome may be lost, (LOH, loss of heterozygosity, discussed below) which may result in the losing a tumour suppressor gene, allowing for uncontrolled cellular growth (26,27).

Microsatellite instability (MSI)

Microsatellites are repeated sequences of normal untranscribed DNA throughout the genome. In case a mismatch repair gene (MMR gene) becomes mutated, as in HNPCC, or silenced by methylation, microsatellites accumulate replication errors and become longer or shorter, i.e. they become instable. In sporadic CRC this can be seen in approximately 15% of cases. The MSI *high* phenotype has minimal numbers of LOH. Patients with MSI seem to have a better prognosis than patients with microsatellite stable (MSS) tumours (28).

CIMP (CpG island methylation phenotype)

The promoter regions of approximately 50% of all genes contain CpG islands. Normally, hypermethylation of these CpG islands may reflect an epigenetic mechanism that reinforces long-term gene silencing. In patients with CRCs of the CIMP trait, abnormal hypermethylation of numerous genes is seen, caused by disrupted regulation of DNA methylation (CIMP). If the methylation occurs at a MMR gene promoter, such as the MLH1 gene, the gene is silenced and the phenotype develops into a MSI type.

LOH

The loosing of one of the two inherited genes of an allel is known as *loss of heterozygosity* (LOH). In tumour cells the alleles that are affected commonly contains a tumour suppressor gene (TSG). If the remaining allele contains a silenced TSG, via promoter methylation or by other mechanisms, or a mutated TSG it renders a total loss of function for that specific TSG, allowing for tumorous growth. In CRC, LOH at chromosome 18q has been associated with a poorer prognosis in stage II patents according to several authors (29-31). However, Carethers *et al* and also other investigators were not able to verify the importance of LOH at 18q as a prognostic marker in stage II CRC patients (32).

Epigenetics

Epigenetics is a broad term used to describe the regulation of gene expression due to mechanisms other than changes in the underlying DNA sequence. The most common of these mechanisms is DNA methylation in a promoter region of a gene, another being histone deacetylation. Regardless of the mechanism, the overall result is silencing of the gene, without subsequent transcription. Both environmental factors and inherited factors contribute to epigenetic changes (33). In colorectal cancer, hypermethylation is commonly seen and is associated with silencing of a number of tumour suppressor genes.

Oncogenes

Oncogenes are mutated or over expressed normal growth-controlling genes. Oncogenes drive the cells to grow, divide, and protect them from programmed cell death, or apoptosis. Oncogenes have been shown to be an important part of the tumorigenesis in CRC (7,34). A few of the principal oncogenes have been discussed below.

KRAS

Ki-ras2 (Kirsten rat sarcoma viral oncogene homolog) is an oncogene that encodes a small GTPase transductor protein called K-ras. The gene is located on the short arm of chromosome 12 (12p). It is mutated in 40-50% of sporadic cases of CRC. The point mutations are seen in codon 12 in most cases but also at codon 13 and 61. It has been shown that K-ras mutations in metastatic disease are a predictor of resistance to Cetuximab (anti-EGFR) therapy, and, furthermore, they are associated with a worse prognosis of disease, as described by Lievre et al, and Font et al (29, 35, 36). Price et al, however, could not show any relations to VEGF and to overall survival in metastatic CRC patients with mutated K-ras (37). K-ras mutations together with P16 methylation further indicate a poorer survival according to Esteller *et al* (38). Fung *et al* and Bouzourene *et al*, could not, however, show the benefit of the K-ras mutation as a prognostic factor (39,40). The prognostic value depends on what kind of K-ras mutation a tumour has according to Winder, *et al*, and Senagore, *et al*,(41, 42).

PIK3CA

The PIK3CA gene encodes the p110 alpha catalytic subunit of P13K (phosphatidylinositol 3-kinase). Mutations in PIK3CA are thought to constitutively activate the AKT pathway, thereby driving cellular proliferation. PIK3CA mutations

have been described in 10-30% of colon cancers (43). And mutations in exon 20 have been associated with poorer prognosis in stage III patients (44). Other investigators showed poorer prognosis in patients with mutated PIK3CA only in patients with wild type (not mutated) K-ras (45).

BRAF

BRAF kinase is a downstream target of KRAS and activates the MAPK (mitogen-activated protein kinase) pathway. The BRAF mutation is linked to CIMP phenotype (46). Tol et al, has shown that in patients with metastatic CRC, a mutated BRAF is a negative prognostic marker (47).

Tumour suppressor genes

The genes that operate to constrain or suppress cell proliferation are called *tumour suppressor genes* (TSG). When these genes are mutated, inactivated, or lost, they result in aberrant cell growth, thereby playing an important role in tumorigenesis. A few of the key tumour suppressor genes are described in the following.

APC

The genetic coding associated with familial adenomatous polyposis coli was identified in 1991 by Groden, *et al*., and is now called the APC gene, encoding a 2843 amino acid peptide(48). This TSG is located on the long arm of chromosome 5 (5q). This location is vulnerable, both LOH at 5q and mutations that inactivates the APC gene are common findings in CRC. Hsieh, *et al*., showed that, in blood serum taken preoperatively in CRC patients, mutations in APC and p53 were closely correlated with lymph node metastasis and the TNM stage. APC gene mutation found in serum in CRC patients is further associated with locoregional metastasis (49).

P53

Mutations in the TP53 (tumour protein 53) gene is common in *all* human cancers. The gene is located on the short arm of chromosome 17 (17p). It is known as "the guardian of the genome" due to its central role in regulating the cell cycle, initiating apoptosis when the cell is under physical stress, inhibiting angiogenesis, and stabilizing the genome. Despite the obvious importance of this protein prognostic studies have not been conclusive. However, mutated p53 in serum is associated with peritoneal metastasis (49). The familial condition of inherited cancers, Li Fraumeni syndrome, has a p53 germ line mutation.

P16

The tumour suppressor gene p16 encodes for a cyclin-dependant kinase inhibitor, p16, which acts like a negative regulator of cell growth and proliferation in the G1 phase of the cell cycle (50). Hypermethylation of the gene p16INK4a in the mucosa in colorectal cancer patients is associated with reduced survival (51).

MGMT

O-methylguanine-DNA methyltransferase (MGMT) is a DNA repair enzyme. Promoter hypermethylation of the MGMT gene is thought to be an early event in carcinogenesis. The role in prognosis has, however, not been clearly established (52,53).

PTEN

The PTEN gene encodes a protein that is a phosphatase and tensin homolog (PTEN). The gene is located on the long arm of chromosome 10 (10q). It regulates the cell cycle and acts as an apoptotic inducer. It negatively regulates the AKT signalling pathway via p13K. Mutation and loss of expression of the gene is seen in both MSS and MSI

tumours (54). Sawai, *et al*, found an association between loss of expression of the PTEN gene and liver metastases in CRC patients (55). Loss of expression might also be a negative predictor to anti-EGFR treatment (56-58). The PTEN gene is a target for the microRNA-21 (59-62).

DCC

DCC (deleted in colorectal carcinoma) is a gene that encodes for a trans-membrane receptor protein. It is located on the long arm of chromosome 18 (18q). It has been shown to induce apoptosis, but whether or not the gene qualifies as a tumour suppressor gene is still under debate. Some studies have shown the potential of DCC as a prognostic marker, whilst others have not been able to show this (63, 64).

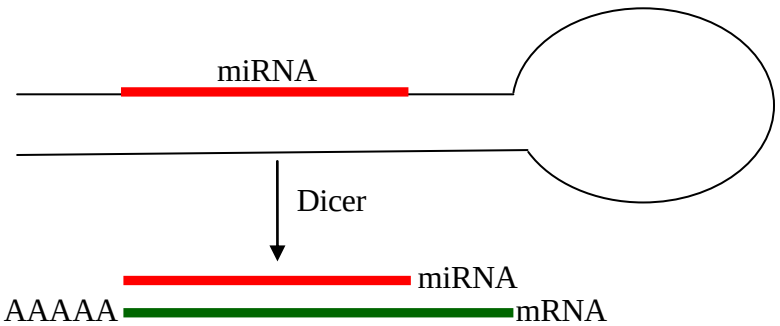
Table 2. Some important oncogenes and tumour suppressor genes involved in CRC. References given in parentheses.

Oncogenes	Prognostic or predictive value found	No prognostic value	**Tumour suppressor genes**	Prognostic Or predictive value found	No prognostic value
K-ras	(29,35,36,38,41)	(37,39,40)	APC	(49)	
PIK3CA	(44,45)		P53	(49)	
BRAF	(47)		P16	(51)	
			MGMT		(52,53)
			PTEN	(55-58)	
			DCC	(63)	(64)

MicroRNA

A microRNA (miRNA) is a small non-coding RNA first discovered by Lee, *et al*, in 1993 (65). To date, there have been more than 900 miRNAs discovered. After transcription, the pre-miRNA is cleaved twice, first in the nucleus by an enzyme called Drosha, and then in the cytoplasm by another enzyme called Dicer (Figure 2). The mature miRNA is approximately 22 nucleotides long (66). The miRNAs regulate many biologic processes by silencing gene expression. They act post-transcriptionally by forming a RISC (RNA induced silencing complex) complex that can bind to the 3´UTR (untranslated region) of a messengerRNA (mRNA). If the match is perfect, the mRNA is cleaved, and if it is imperfect, the translation is inhibited (67). The small miRNAs are more stable than mRNAs and are therefore promising candidates as biomarkers of disease. Thus, they can be extracted and analysed from frozen as well as embedded tissue and from serum/plasma after years of storage. MiRNAs that target TSGs are called oncomiRs due to their role in carcinogenesis. Alterations of the expression of specific miRNA have been described for many tumour types, including CRC (68-71). Lawrie *et al*, was the first to describe miRNAs in serum in cancer patients (72). A systematic review and meta-analysis concluded that elevated miR-21 expression did successfully predict poor survival in patients with a variety of carcinomas (73).

Figure 2. The hairpin structured miRNA is cleaved by the enzyme Dicer and as a mature miRNA it binds to a messenger RNA and inhibits translation.



Other prognostic markers

The SMAD proteins are intracellular components of the TGF-beta signalling pathway. Loss of SMAD activation and/or expression occurs in approximately 10% of CRCs. This subset is associated with a poor prognosis (74). Carcinogenic antigen (CEA) is a tumour derived protein that has been used as a serological prognostic marker for many years. It may be most useful in the early postoperative period to help determine macroscopically radical tumour resection, as well as for the detection of early recurrence/metastases (75,76)

Clinical routine

Histopathological routine examinations and TNM classification remains the mainstay in our prognostic abilities. In the majority of tertiary care facilities with treatment centres for CRC, CEA levels are taken as a routine investigation on a regular basis. In additional, pathologists utilise immunohistochemistry (IHC) to detect the Kras status if the patient is a candidate for anti-EGFR treatment. For patients with suspected HNPCC, a MSI status is also examined. There is, however, a need to investigate and develop genetic analyses in clinical practice as a routine to improve prognosis and the allocation of proper adjuvant therapies on a more individualised basis.

2 Aims

Study I
To evaluate the intratumoural distribution of some genetic events of prognostic interest such as LOH, methylation of genes and point mutations in CRC.

Study II
To evaluate K-ras mutations, and thereby to examine the possibility to detect micro metastases in normal appearing lymph nodes, in stage II CRC patients and to correlate those to prognosis.

Study III
To screen for and determine the impact of microRNA expression levels in tumour tissue on the prognosis of CRC patients.

Study IV
To evaluate relative changes in the expression levels of three miRNAs (miR-21, miR-133b and miR-185) in serum prior to, and after, radical surgery for CRC, and to further correlate those to prognosis.

3 Material/Methods

Paper 1

17 consecutive patients with CRC were included. Clinical data is shown in Table 3.

Table 3. Clinical data

Gender	Female	9
	Men	8
Age	Range	37-88
	Median	76
Stage	II	10
	III	7
Localisation		
Right colon		8
Left colon		5
Rectum		4

The tumours were divided into 3 mm cubes and stored at -70 degrees Celsius. DNA was extracted using a Qiagen kit. Polymerase chain reaction was conducted with primers and tested for LOH in different loci and estimated according to Cawkwell et al (77). Microsatellite instability appeared as bands of varying sizes in comparison to normal DNA, was determined by automated analysis.

Kras was amplified between codon 9 to 30, and the mutations at codon 12 and 13 were detected using temperature gradient gel electrophoresis (TGGE). To detect the methylation status for p16 gene, and MGMT gene, specific primers were used as shown in table 4.

Table 4. Methylation-specific primers for p16 and MGMT

	Methylation specific primer-sense	Methylation specific primer-antisense
Unmethylated p16	5`-TTA TTA GAG GGT GGG GTG GAT TGT-3`	5`-CAA CCC CAA ACC ACA ACC ATA A-3`
Methylated p16	5`-TTA TTA GAG GGT GGG GCG GAT CGC-3`	5`-GAC CCC GAA CCG CGA CCG TAA-3`
Unmethylated MGMT	5`-TTT GTG TTT TGA TGT TTG TAG GTT TTT GT-3`	5`-AAC TCC ACA CTC TTC CAA AAA CAA AAC A-3`
Methylated MGMT	5`-TTT CGA CGT TCG TAG GTT TTC GC-3`	5`-GCA CTC TTC CGA AAA CGA AAC G-3`

Positive and negative controls were compared with the PCR products on 2,5 % agarose gels.

Paper II

Ninety-nine Stage II CRC patients who underwent surgery during 1978-82 were included; the gender distribution was 56 men and 43 women (n=99); 27 had right-sided CRC, 5 had transverse CRC, 35 left-sided CRC and 32 had rectal cancer. The age range was 32-87 years, with a median age of 71 years. After DNA extraction from the tumours and the lymph nodes from the embedded tissues, Kras status was evaluated using the TGGE (as in paper I). Furthermore, a mini meta-analysis was done to compare and assess literature data in the field.

Paper III

Fifty patients (26 men and 24 females) with CRC were included. The median age was 75 (range 37-87). 15 had right-sided colon cancer, 18 left-sided colon cancer and 17 rectal cancer. The tumours were immediately frozen and stored at -70 degree Celsius. Total RNA isolation was performed using the mirVana miRNA Isolation Kit (Applied Biosystems/ Ambion, Austin, TX). The SYBR Green-based qRT-PCR miRNA array platform was used for the profiling of 10 patients with distinct survival patterns (5 patients < 50 months, 5 patients ≥ 50 months). Quantitative real-time PCR was performed using the Power SYBR Green Master Mix on the 7900HT Real-time PCR System (Applied Biosystems) employing QuantiMir universal reverse primers and miRNA-specific forward primers. All data values were normalized by geometric means of three different reference genes (Human U6, RNU43 and U1), and relative quantification was calculated as $2^{-\Delta CT}$. Normalized miRNAs with <20% missing values were included in subsequent analyses for hierarchical clustering based on un-centered correlation and complete linkage using Cluster 3.0 (78) and visualized using Java TreeView. Significance Analysis of Microarrays (SAM) was used in order to identify the most significant miRNAs associated with survival. *p*-values were obtained for the Cox score statistics using the χ^2 distribution.

Selected mature miRNAs were quantified using commercially available TaqMan qRT-PCR assays (Applied Biosystems) and a 7900HT Real-Time PCR System (Applied Biosystems). cDNA was synthesized from 100 ng RNA using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and used for quantification of *miR-133b* (ID 002247), *miR-185* (ID 002271), *miR-320b* (ID 002844), *miR-21* (ID 000397), *miR-663b* (ID 002857), *miR-892b* (ID 002214) and *miR-615-5p* (ID 002353), *RNU6B* (ID 001093) and *miR-16* (ID 000391). Due to the low or

undetectable level of *RNU6B* expression, the expression of miRNAs was normalized to *miR-16*. All reactions were performed in triplicate, and relative expression levels were determined with the ΔC_T method and reported as $2^{-\Delta CT}$.

Tumour samples were classified into two different groups, based on high or low expression of each miRNA according to the median level. The interrelationship of miRNAs with survival was studied using Kaplan-Meier plots, while significant differences between curves were evaluated using log-rank test. The significance of individual miRNA expression in correlation with metastasis and other clinical characteristics, including age, gender, stage and recurrence, was studied using Fishers' exact test. All *p* values obtained in this study were 2-tailed, and *p*-values < 0.05 were considered as significant. All statistical tests were performed in Statistica 8.0 (StatSoft, Inc., Tulsa, OK), unless otherwise stated.

Paper IV

Sixteen consecutive patients with CRC and one patient with high grade dysplastic adenoma were included. Blood serum was collected immediately prior to surgery and on the 30th postoperative day, and stored at -70°C. All operations were so called R0 (radical operations).

Total RNA isolation was performed according to following protocol: 250 uL of each serum sample was mixed with 500 uL lysis buffer (mirVana miRNA Isolation Kit, Applied Biosystems/ Ambion, Ausin, TX) and 800 uL acid phenol: chloroform (Ambion, Austin, TX), vortexed for 30 sec, and centrifuged at 16000 rcf for 10 minutes at room temperature. The aqueous phase was mixed with an equal volume of acid phenol: chloroform and centrifuged at 16000 rcf for 10 minutes for two times at room

temperature. The resulting mixture was precipitated with a 0.1 volume measurement of 3 M NaCl, 2 uL glycogen (1 mg/mL) and 2.5 volume of 100% ethanol at -20C° for at least 1 hour. After centrifugation at 4C° at max speed for 30 minutes, the pellets were air-dried at room temperature. The pellets were resuspended in 20uL elution buffer. RNA concentrations were measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Three miRs (miR-133b, miR-185 and miR-21) were analysed using TaqMan qRT-PCR, as in paper III.

4 Results

Paper I

17 CRC tumours were divided into small cubes and the distribution of the chosen genetic events were examined. The distribution of the allelic loss, methylation and K-ras-mutations in the different biopsies is shown in table 5 and 6. The K-ras mutation is homogeneously spread throughout the tumour, while the methylation, and in particular the LOH, are randomly distributed. However, LOH seems to be almost mandatory found in all tumours.

Table 5. The number of biopsies harbouring K-ras mutations, methylation of the p16 gene or methylation of the MGMT gene per the total number of biopsies taken from each tumour.

Patient #	K-ras-mut/tot	p16 meth/total	MGMT meth/total
1	0/25	11/25	25/25
2	23/23	25/25	0/25
3	0/27	15/19	18/20
4	0/20	16/20	16/20
5	1/20	9/20	5/20
6	0/33	12/19	1/19
7	0/22	21/21	3/21
8	15/17	18/19	13/18
9	0/23	19/19	17/19
10	17/17	19/19	5/19
11	0/27	4/20	19/20
12	17/18	17/17	12/13
13	26/26	1/19	0/19
14	0/20	19/20	18/20
15	0/30	0/20	19/20
16	19/19	8/20	0/20
17	20/20	9/20	17/20

Table 6. The number of biopsies harbouring LOH per the total number of biopsies taken from each tumour. H = homozygotes for the chosen microsatellites; MI = microsatellite instability; ND = not done.

Patient #	**LOH 18q**		**LOH 17p**		**LOH 5q**	
	D18S58	D18S67	D17S796	D17S1832	D5S299	D5S495
1	1/36	25/36	19/37	33/37	2/40	2/40
2	H	12/25	1/40	H	6/10	ND
3	2/24	8/26	H	H	14/28	12/20
4	0/23	10/23	6/10	10/18	0/40	2/40
5	17/40	6/39	H	H	12/40	21/40
6	8/20	14/20	14/20	H	H	H
7	1/42	4/42	H	8/19	6/20	12/20
8	6/18	H	4/18	12/18	19/38	H
9	2/34	8/34	H	2/48	8/35	6/35
10	H	12/20	11/20	6/20	H	14/20
11	16/19	7/17	2/38	3/38	15/19	H
12	15/18	8/18	14/18	H	11/17	12/18
13	5/18	0/18	2/38	H	2/18	MI
14	H	8/19	6/20	H	1/20	MI
15	20/20	19/19	H	19/19	H	17/19
16	10/18	14/18	17/20	19/20	H	0/19
17	15/20	20/20	16/20	H	16/20	20/20

Paper II

99 patients with Stage II CRC underwent radical surgery. K-ras status from tumours, lymph nodes and normal mucosa were examined. 34/99 (34%) patients were found to harbour K-ras mutations within cancerous tissue areas, consisting of invasive cells microscopically. 10/34 (29%) of these were K-ras positive in the locoregional histologically normal appearing lymph nodes. Conversely, all lymph nodes with K-ras mutations correlated to a tumour harbouring K-ras mutations. 7/10 patients with Kras positive lymph nodes had a relapse of the disease and died within 60 months from the time of primary surgical intervention. Previous studies have shown similar results when testing for overall effect and calculating risk ratio (79-81). These results, together with our results, show that the total risk of recurrence of the disease was found to be more

doubled (2,49) and this result for overall effect is statistically significant (p=0.002). The occurrence of K-ras mutations in different specimens and the risk of developing metastatic disease are shown in Figure 3. The overall effect and risk of death of micro metastasis within the lymph nodes, according to recent studies, and including ours, is shown in Figure 4.

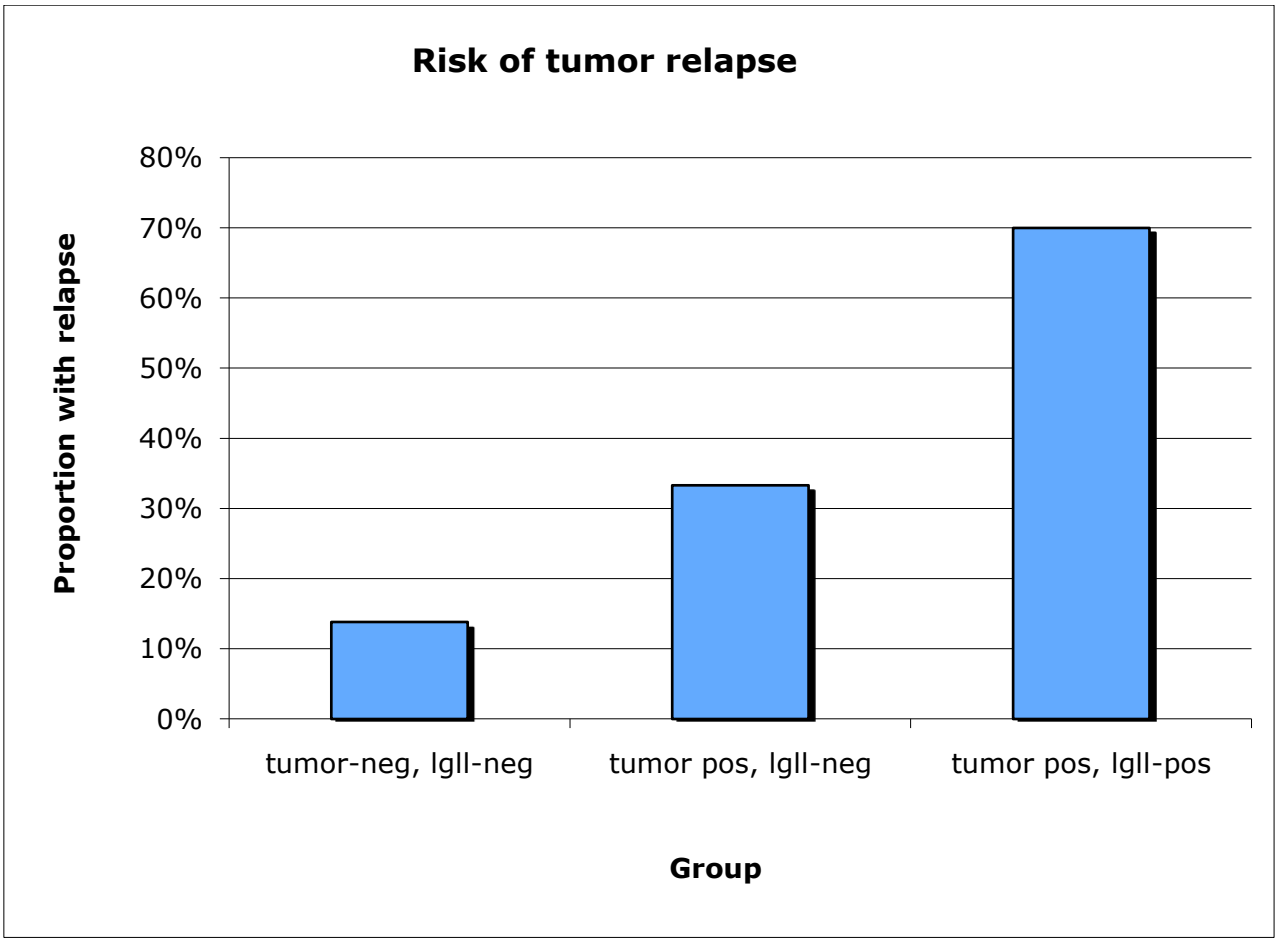


Figure 3. The proportion of relapses among patients with or without K-ras mutations in tumour and locoregional lymph nodes.

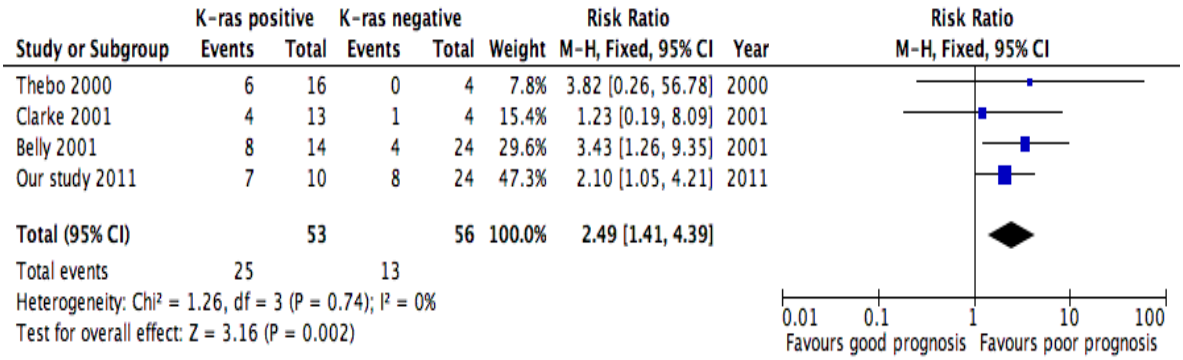


Figure 4. Risk of relapse in colorectal cancer in relation to K-ras mutation positivity in the tumour and in the lymph nodes, respectively

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Paper III

50 patients with CRC were analysed in order to detect deregulated miRNA expression levels in tumour tissue. At the time of follow-up, 23 patients had succumbed to the disease, 14 had died of unknown or other causes and 13 were still alive. In comparison to the long term survival group of patients, SYBR-green test detected 7 over-expressed and 356 under-expressed miRNAs in the short survival group.

Three over-expressed (*miR-185*, *miR-320b* and *miR-663b*) and three under-expressed miRNAs (*miR-133b*, *miR-615-5p* and *miR-892b*) were selected for the TaqMan test. In addition, we also assessed the expression level of *miR-21* in the validation cohort because high expression of *miR-21* has previously been associated with poor survival in CRC (73). However, we did not find any significant difference between the two groups based on our screening data for *miR-21*.

Using Kaplan-Meier survival and log-rank analyses, we evaluated the association with overall survival for each individual miRNA expression. Patients with high expression of *miR-185* (p=0.001; log-rank test) and low expression of *miR-133b* (p=0.028; log-

rank test) were found to have a significantly shorter survival (Figure 5). They were also associated with metastasis.

No significant association was found for the other five miRNAs tested.

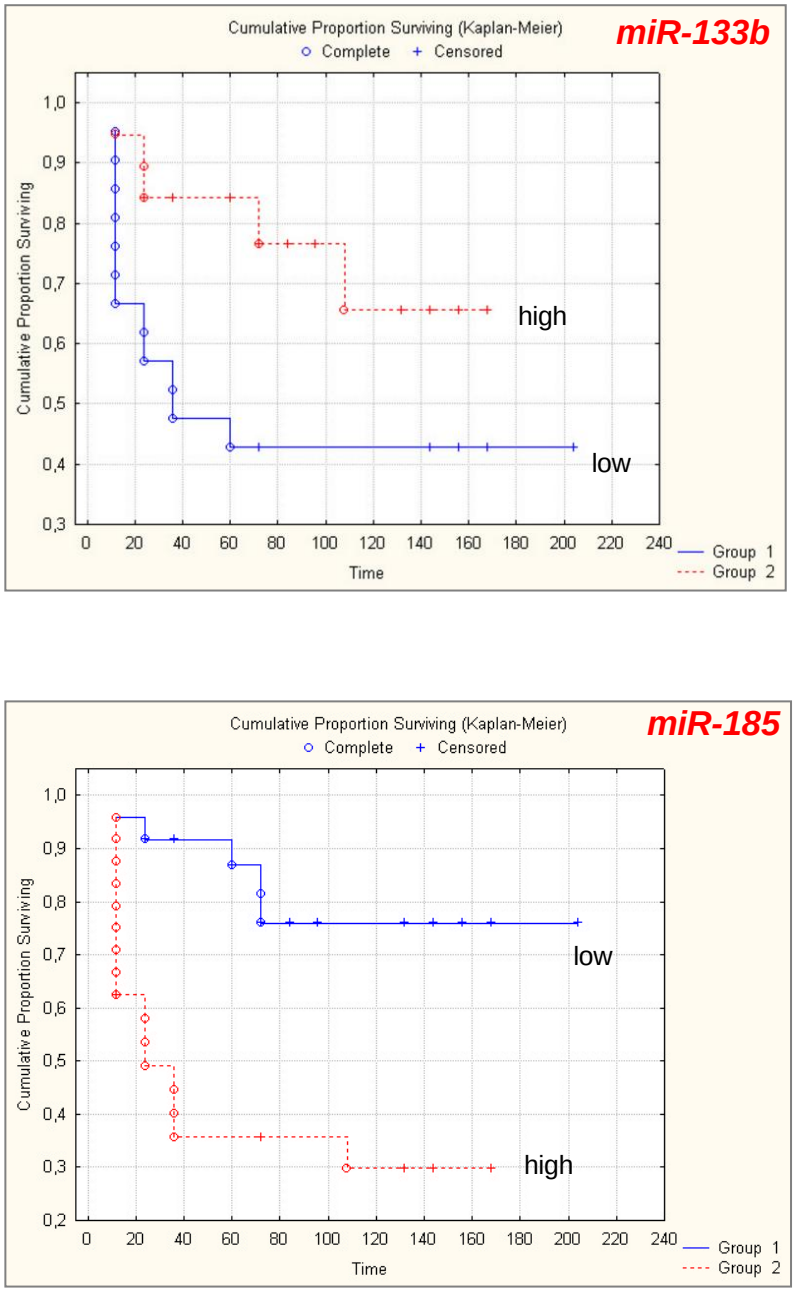


Figure 5. Kaplan –Meier survival plots of patients with high (red) and low (blue) expression of miR-185 (*p*=0.00045, log-rank test) in CRC tumour tissue.

Paper IV

17 CRC patients were included and serum was collected prior to and after surgery to evaluate the relative changes in three miRNAs expression levels. Clinical and follow-up data is shown in Table 7.

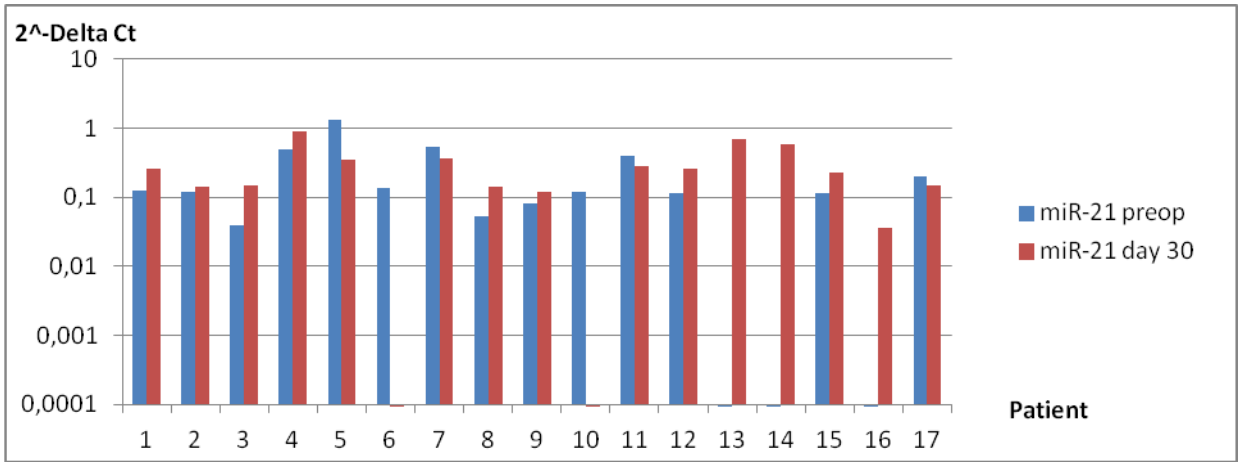
Patient	Age	Gender	Tumour site	Stage	Death Disease related	Death Disease unrelated	Adjuvant Chemo	Relapse
1	77	F	Left colon	3			No	No
2	80	F	Right colon	2			No	No
3	78	M	Sigm/rectal	2		X	No	No
4	72	F	Right colon	2	X		No	Liver
5	67	M	Right colon	3		X	No	No
6	62	F	Right colon	3			Yes	No
7	77	M	Left colon	2			No	No
8	71	F	Left colon (lap)	2			Yes	Local recurrence 2005
9	65	M	Right colon	3			No	No
10	61	F	Rectal	1			Yes	Lung 2004
11	74	F	Right colon (lap)	2		X	No	No
12	76	F	Left colon	2	X		Yes	Lung 2004
13	68	M	Left colon (lap)	3			Yes	No
14	77	M	Right colon	3			Yes	No
15	71	M	Left colon	adenoma			No	No
16	76	M	Rectal	3			Preop rad	No
17	28	M	Right colon	2			No	No

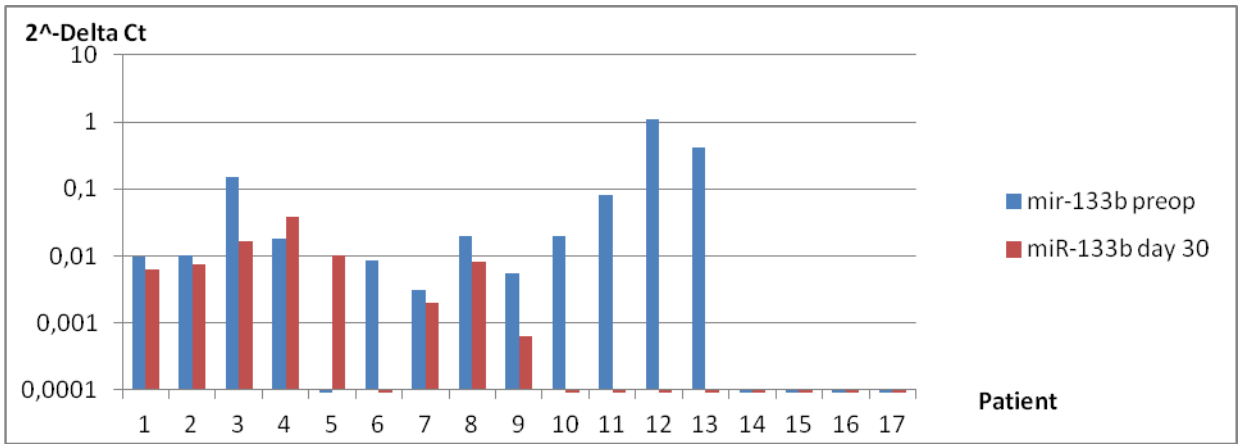
Table 7. Clinical information and follow-up data

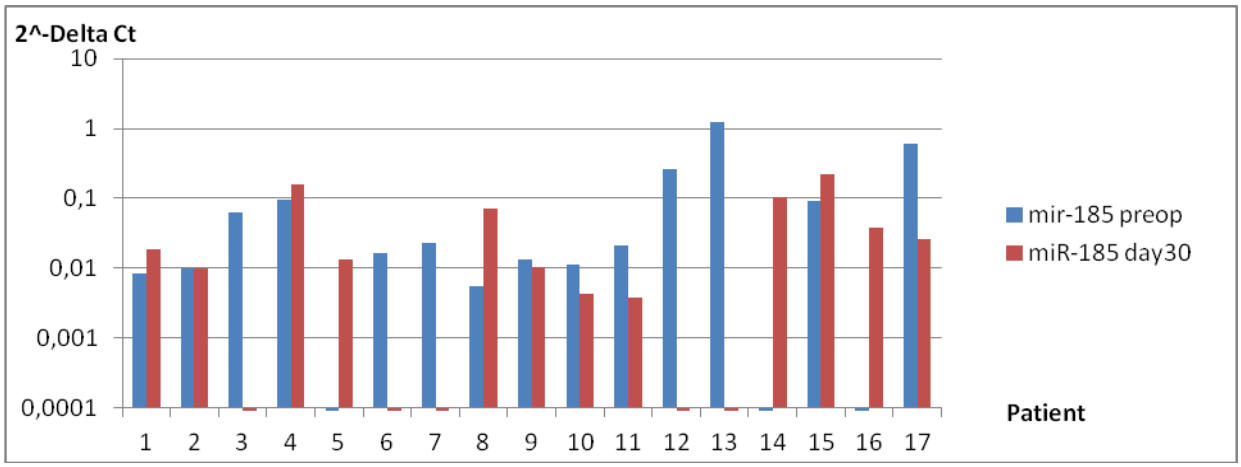
All the three miRNAs evaluated were detectable in the majority of all serum samples (75/102; 72 %). Of these, miR-21 was the most prominent in terms of serum levels. However, for this specimen, there was no effect seen upon removal of the tumours in this series of patients as a slight, non-significant increase were detected on the 30th postoperative day as compared to the baseline (mean ±SEM 0.227± 0.080 preoperatively vs. 0.274± 0.060 postoperatively) .

miR133b increased in one patient postoperatively. This patient later developed liver metastases and died from disseminated disease. Another patient developed low levels of miR-133b postoperatively from undetectable levels preoperatively; this patient died shortly after day 30 from surgical complications. All other patients (15/17) displayed decreases, or undetectable levels (4/17 preoperatively; 8/17 postoperatively) of miR-133b. In the patient cohort as a whole, there was a significant effect of tumour resection on miR-133b ($p=0.027$).

Figure 6. miRNA levels preoperatively (blue) and at day 30 (red)







5 Discussion

The results of paper I indicate an important difference in the rates and characteristics in the occurrence of point mutations of the K-ras gene in comparison to both allelic imbalances at chromosome 5, 17, and 18 and methylation of the p16 and MGMT genes. Allelic loss and methylation were demonstrated to occur regularly and appeared to be a prominent finding in advanced CRC tumours, while K-ras mutations occurred in approximately half of the tumours in the mutated form and were conserved as the wild-type gene in nine of the seventeen studied cases. The standard methodology for characterizing genetic changes in CRC involves analysis of very limited areas of the tumours, using one or a few biopsies (39, 40, 82-86). However, these tumours generally display a morphologically mosaic-like pattern, representing different intra-tumour sub-clones of cancer development. Thus, biopsies for DNA aberrations that are heterogeneously distributed throughout tumours as shown here could result in uncertain and even clinically irrelevant data. This may also contribute to the variability in the importance of certain markers as indictors of prognosis.

K-ras mutations, when present, are found early in the adenoma – carcinoma sequence (25) with a proportion of K-ras mutations in studies of colonic adenomas similar to those of carcinomas (29, 39, 40, 87, 88). From our results, with only 3/17 tumours not containing homogeneously mutated or wild-type K-ras, one may conclude that this early event appears to be strikingly preserved throughout clonal expansion and tumour progression in CRC.

At present the decision to use anti-EGFR therapy in metastatic CRC is based on the K-ras status and this study is supportive of the use of a single biopsy to predict treatment responsiveness (18-20).

In the second study, we were able to detect the occurrence of mutated K-ras in DNA from microscopically normal loco-regional lymph node tissue in stage II CRC patients. In this case it might be harder to use just one biopsy from the lymph node, since there may be just a few tumour cells that contain the mutation. TGGE is considered to be a sensitive method and therefore a useful tool to detect a few cells with mutations among the abundance of K-ras wild type cells. In our study, this finding of occult metastases correlated to a worsened prognosis in terms of metastatic relapse and disease specific mortality.

In routine clinical practice, identification of metastatic cancer cells in regional lymph nodes by microscopic investigation is one of the most significant prognostic factors in the staging process (89-91). Stage II cancer, representing the finding of pathologically normal loco-regional lymph nodes, usually correlates to a relatively good prognosis, though approximately 20-30 percent will eventually die from metastatic disease (89).

Mutation of the K-ras gene as a disease marker in healthy lymph nodes and detected in 10/99 of the cases we studied, appears to be predictive of an increased risk of developing disease recurrence and disease related mortality. 7 out of the10 patients (70%) later developed recurrent disease; either through local relapse or metastases. The lack of statistical significance ($p=0.07$) may be due to the relatively small number of patients and the small number of lymph nodes. However, by combining recent and similar research in the field of occult lymph node disease, it is possible to demonstrate a significantly higher risk ratio (2,5 times, $p=0.002$) of developing metastases and relapse in the disease (79-81).

34 of the 99 CRC patients that we analysed in our study had K-ras mutations in the invasive cells of the tumour specimen. This frequency is similar to that of previously reported for K-ras mutations in CRC tumours (29, 80, 92, 93). Our results suggest that adding molecular techniques may add sensitivity to the microscopic examination and may be a valuable supplement in the efforts of sub-classifying patients with stage II CRC. 65 of the 99 patients did not show K-ras mutations in their tumour specimens. However, by the addition of other genetic analyses in locoregional lymph nodes we may increase our ability to identify subgroups of Stage II CRC patients which in turn could benefit from adjuvant treatment.

DNA is stable and therefore suitable as a biomarker but there seems to be a difference between the representativeness of the different DNA and chromosomal changes in the tumour. MicroRNA is, as mentioned previously, a recently discovered small RNA that appears to have potential to become useful as a biomarker; due to its stability and free circulation in blood.

In the third study we focused on miRNAs. The deregulation of two miRNAs (*miR-185* and *miR-133b*) correlates with patient survival and metastasis, suggesting that these miRNAs (or their targets) may have prognostic implications in CRC. In tumour tissue, *miR-185* has been found to inversely correlate to PTPN13 (protein tyrosine phosphatase, non-receptor type 13), a putative tumour suppressor gene that can suppress cell growth and induce apoptosis (94, 95). However, the role of *miR-185* is somewhat complex as it appears to target different genes in different cell types, contribute to different biological processes, and may even act in a tumour suppressing way in certain cancer cell lines (96).

Low expression of *miR-133b* is associated with poor survival and metastasis among CRC patients in our study. *miR-133b* is down-regulated in several other cancer types, including colorectal cancer,(97), lung cancer,(98) bladder cancer,(99) gastric cancer,(100) esophageal squamous cell carcinoma (101) and squamous cell carcinoma of tongue(102). In addition, *miR-133b* has been reported to have a prognostic potential in bladder cancer (103).

The expression of *miR-21* is reportedly increased in many tumour types, including CRC, and its up-regulation has been associated with tumour cell invasive abilities, poor survival and the suppression of tumour suppressor proteins (104, 105). Interestingly, Nielsen *et al.* observed that the expression of *miR-21* was predominantly found in fibroblast-like cells located in the stromal compartment of the colon tumours. *miR-21* has also been related to remodelling of extra cellular matrix and associated with cellular motility and key enzymes in extra cellular matrix breakdown (106). Since its emergence as an important regulator in the control of many cell functions, *miR-21* has been extensively studied in various fields including angiogenesis, aging and wound healing (107).
However, we did not observe a significant difference in expression levels between the long and short survival groups of CRC patients using both SYBR-green and TaqMan qRT-PCR assays.

Circulating miRNAs exist and are stable in human serum and plasma and are much less susceptible to degradation of nucleases than RNA. These properties make them highly attractive as serum markers (108). We therefore investigated *miR-185, miR-133b* and *miR-21* as potential novel non-invasive serum biomarkers for the prognosis of CRC patients following surgery.

In the forth study we did not find any correlation between the serum baseline levels of *miR-21, miR-133b,* or *miR-185* and the risk of recurrent disease in CRC patients. In addition, there was no correlation between intra-individual changes in serum levels and disease outcome. The levels in serum of *mir-21* were not affected by radical tumour resection. However, there was a significant decrease in the level of *miR-133b* among the patients following surgery, declining or being undetectable in 15/17 patients. Furthermore, an overall reduction for *miR-185* was also seen.

Circulating tumour-specific nucleic acids as biomarkers for the early detection of CRC, and other tumours, would present a patient-friendly, non-invasive alternative to current screening procedures. This method could also serve as a convenient predictive tool in judging the effects of treatment and the monitoring of the individual patient's need of surveillance. In a previous report, the prognostic value of serum analysis of circulating mutated K-ras in CRC patients was evaluated (109). A wide array of investigations looking at the value of mutational analysis of DNA, mRNA and epigenetic events in plasma and serum have been studied, though the impact on clinical implications is as yet unclear (110). The different mRNA targets and the function of many miRNAs have been intensively explored over the last years, with emphasis on their role in neoplastic development. Each miRNA may potentially regulate the expression of tens to hundreds of protein-coding genes, though different miRNA are specific to different tissues. In both our studies, *miR-21* levels were not significantly deregulated and did not show any prognostic value. This finding strongly indicates that a substantial part of this miRNA in the serum of CRC patients does not originate from loss or secretion from the cancer cells. Thus, the results may well indicate that a multitude of cells, including fibroblasts, involved in the postoperative healing and remodelling processes may be the source of *miR-21* during serum sampling postoperatively. The absence of serum from healthy

controls having undergone surgery for benign disease in this study precludes conclusions on the relative role of tumour cells preoperatively and fibroblasts and other cell types involved in the healing process postoperatively.

The mean postoperative levels in serum of *miR-185* are decreased in this study. This may reflect a major depletion of the cells and source of *miR-185* origin after radical surgery. The ambiguous biological role of *miR-185* and the fact that a few patients (4/17) showed elevations in their serum levels postoperatively indicates that larger studies are needed to explore the prognostic significance of *miR-185* in serum.
The expression of *miR133b* in serum is significantly decreased with tumour removal in this patient cohort. However, the role of *miR-133b* and its targets in CRC progression remains to be further investigated.

6 Conclusions

- LOH 5q, 17p, and 18q, and methylation of MGMT and p16, are unevenly distributed throughout CRC tumours and of limited value as prognostic markers if derived from single biopsies.
- K-ras mutations are evenly distributed throughout CRC tumours, in line with the concept that they appear early in tumour development.
- K-ras mutations can be found in histologically healthy lymph nodes and seem to predict a worse prognosis in Stage II patients with this finding.
- Low expression of miR-133b and high expression of miR-185 in CRC tumours are associated with poor survival and the development of disseminated disease.
- The level of miR-21 in blood serum is not affected by radical surgery for CRC.
- The level of miR-133b in blood serum is significantly reduced after radical surgery for CRC.

7 Future perspectives

The pathological anatomical diagnosis (PAD) continuously improves. Still, there is a need for better tools to assess prognosis and direct appropriate treatment to the right patient. A future way to meet these needs may be the use of standardized set of molecular diagnostic analyses. The work in this thesis points out some of the molecular events surrounding tumorigenesis in CRC and further suggests the potential use of certain molecules as biomarkers, either for diagnosis and prognosis, or as an aid in assessing the need for adjuvant therapy. Though a fair amount of pre-clinical studies have been conducted, this has yet to be translated into clinical practice. The solution is multifactorial. The current evidence level is rather poor due to the limited number of patients in several studies, the lack of sub-groups, and limited number of biopsies. These factors all lead to contradictory results and low reproducibility. The costs and time-consuming laboratory work also make it difficult to integrate molecular analyses as a clinical routine, despite promising data. At present, immunohistochemistry (IHC) is used routinely to detect K-ras mutations in metastatic CRC to predict response to anti-EGFR treatment. However, IHC has limitations in sensitivity (111) and can presumably not be used to detect abnormal K-ras in normal appearing lymph nodes.

Malignant cells follow a Darwinian selection and one key question is which molecular events are critical for the tumour to metastasize. To understand this, further molecular studies based on clinical material and outcome data are needed. Since cancer in many ways is a heterogeneous disease, many questions cannot be answered through studies of standardized in vitro cell lines, and thus there is a need for translational collaborative efforts between preclinical scientists and clinicians. Metastases may be more homogenous than the primary tumour due to clonal expansions, but on the other hand a metastasis is aggressive and the turn-over rate is high, making it likely that the mutation

rate is also high. In our first paper we have demonstrated the need to examine the whole tumour to evaluate interesting genetic or molecular events as potential biomarkers. This needs to be further characterized also in metastases.

In breast cancer surgery, the use of sentinel nodes is very important, but this far sentinel node studies in CRC have not been without contradictions. We have shown that the findings of micro metastases in healthy lymph nodes (indirectly by looking at K-ras mutations) indicate a worse prognosis in CRC patients. Since the micro metastases can easily "drown" in different analytic approaches, as the majority of the cells are unaffected, the challenge is to find a sensitive and specific tool that is not too costly and time-consuming. There is a need to further evaluate the detection of sentinel nodes also in CRC, and perhaps blue staining is enough (112) and would make it easier to detect micro metastases.

MicroRNA is a promising potential biomarker. In a near future, the development of high through-put techniques based on microchips may become available detecting different expression profiles. To be able to properly utilize these tools, further development of our ability to correlate these findings to outcome will be needed. Thus, as clinicians, we will need to establish standard patients sub-groups based on molecular tumour analyses and evaluate how these groups behave in relation to prognosis and individualised treatments.

The reasons why a biomarker becomes over- or under-expressed are multifactorial and still in general often unresolved. It may be due to loss or gain of parts of a chromosome, epigenetic silencing, single nucleotide mutations or other mechanisms, in turn causing deregulation. For a clinician the question is always how we can apply new basic

knowledge in clinical practice. Can we use it as a diagnostic, prognostic or a predictive marker that is both sensitive and specific? Can we develop an anti-drug that is safe and without side-effects? How much would it cost and how time-consuming would it be to use it? How many patients would benefit from using this new biomarker? There are still many unanswered questions. In summary, this thesis is a small piece in the large puzzle and may bring us one step closer to finding the answers.

Swedish summary/Svensk populärvetenskaplig sammanfattning

Tjock- och ändtarms cancer är en mycket vanlig sjukdom i västvärlden. I Sverige är det den tredje vanligaste cancer sjukdomen för män och kvinnor. Nästan hälften av dem som insjuknar dör på grund av sjukdomen. Behandlingen består av operation och i vissa fall cellgifter och strålning. För att ta reda på vilket stadium patienten är i görs utredningar med hjälp av röntgen undersökningar före operationen. Efter operationen görs mikroskopisk undersökning av preparatet och då får man en säkrare stadieindelning. Stadium II betyder att det inte finns någon mikroskopiskt synlig tumörväxt i närliggande lymfkörtlar som tas med preparatet. I stadium III finns det växt i lymfkörtlar och det betyder oftast att patienten blir aktuell för cellgiftsbehandling. Tyvärr är denna indelning inte hundra procent säker och det innebär att vissa i stadium II inte får cellgifter, fast det skulle behöva det, samt att vissa med stadium III får cellgifter fast de inte behöver. För att kunna utveckla en säkrare diagnos, prognos och även kunna individualisera tex cellgiftsbehandlingen, behövs fler genetiska analyser, som kan ge mer exakta svar. Denna avhandling består av 4 olika arbeten som har försökt utveckla den genetiska analysen vid tjock- och ändtarmscancer (kolorektal cancer).

I arbete I, analyserades tumörer från 17 patienter som opererats för kolorektal cancer. Tumörerna delades i mycket små bitar och undersöktes på förekomsten av genetiska förändringar. De förändringarna var mutationer i en oncogen (gen som driver tillväxt av cell, kan liknas vid en ”gaspedal”) som heter K-ras, metylering av 2 olika gener samt förlusten av vissa delar av kromosomer. Metylering innebär att en gen blir ”tystad” och

inte längre uttrycks (kan liknas vid att ett hänglås ”låser” genen). Analyserna visade att K-ras mutationerna var jämnt spridda i hela tumören, medan de andra förändringarna var mycket ojämnt fördelade i tumören, men nästan alltid till sist hittades i någon liten bit. Detta innebär att man kan lita på en liten biopsi (provbit) när man letar efter K-ras mutation, men när det gäller andra genetiska förändringar måste man kontrollera förekomsten noggrant först. Detta är viktigt, då man har visat att en typ av cellgift inte har effekt om man har K-ras mutation.

I arbete II, undersöktes lymfkörtelvävnad och tumörvävnad från 99 patienter som var opererade för kolorektal cancer. Man hade bedömt att lymfkörtlarna var friska när man undersökte dem mikroskopiskt. Förekomsten av K-ras mutationer analyserades både i tumörerna och i lymfkörtlarna. Resultatet blev att 34/99 hade K-ras mutationer i tumören och av dessa hade 10 stycken det även i lymfkörtlarna. 7 patienter av dessa 10 (70 %) fick tillbaka sjukdomen i någon form (antingen lokalt eller som metastas (dottertumör) i lever eller lunga). Vid jämförelse av tidigare liknande studier noterades att det föreligger en ökad risk för återfall, dvs en sämre prognos hos de patienter med mutationer i lymfkörtlar som mikroskopiskt sett ser friska ut.

I arbete III, undersöktes 50 patienters tumörer med avseende på så kallade mikroRNA. MikroRNA bildas från DNA och därefter påverkar mikroRNA generna genom att binda till messenger RNA (RNA är ”modell” för protein byggande). Det leder till att proteinet i fråga inte kan byggas. Först undersöktes 10 patienter, där 5 hade dött tidigt och 5 fortfarande levde efter sin operation. Några mikroRNA upptäcktes som hade olika mängd i de 2 grupperna. 6 stycken av dessa valdes ut och nivån hos dessa kontrollerades hos alla 50 patienter. Resultaten visade att mikroRNA-133b var lågt och mikroRNA-185 var högt hos de med dålig prognos= kort överlevnad.

I arbete IV undersöktes mikroRNA-133b, mikroRNA-185 och även mikroRNA-21 i blodet hos 16 patienter med kolorektal cancer och 1 patient med adenom (polyp) i tjocktarmen. Blodprov togs precis före operation och 30 dagar efter operation. Ingen förändring kunde noteras avseende nivåer av miR-21 före och efter operation, men miR-185 hade tendens att sjunka och miR-133b sjönk signifikant efter operation. Ingen korrelation till prognos kunde ses. Bedömningen blev därmed att miR-133b samt miR-185 kan var intressanta ur biomarkör synpunkt, men att miR-21 sannolikt utsöndras från andra celler än tumörceller, t.ex. bindvävsceller.

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