Capillary electrophoresis in analysis of DNA variations in rectal cancer.

A thesis for the doctor philosophiae degree

by

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Oslo, February 2007

Series of dissertations submitted to the
Faculty of Medicine, University of Oslo
No. 516

ISBN 978-82-8072-436-6

Cover: Inger Sandved Anfinsen.
Printed in Norway: AiT e-dit AS, Oslo, 2007.

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Acknowledgment

This work has been carried out in the Department of Surgical Oncology at the Norwegian Radium Hospital.

I would like to express gratitude to my scientific supervisor Per Olaf Ekstrøm for introducing me to the field of cancer research and for invaluable and patient help with the studies and manuscript preparation.

I highly appreciate the collaborations with all my co-authors. Special thanks are given to Johan Wiig for supplying tumor material, lavage fluid, clinical data and comments on the manuscripts. Jens Bjørheim for helping with various manuscripts, and sharing fruitful discussions. Stein Larsen for supplying tumor material and lavage fluid.

I thank the head of the department Karl-Erik Giercksky for research ideas and providing the research facilities and for being my link to the Faculty of Medicine.

I wish to thank present and former members of the Surgical research group David Hinselwood (for comments on the manuscript), Karen-Marie Heintz (technical instrumental support and comments on several manuscript), Torveig Weum Abrahamsen (technical assistance and comments on the manuscript). Special thanks go to all the people in Gustav Gaudernack's research group for creating a great scientific and social environment and for their encouragement and support.

Finally, I would like to thank my family for always being there for me.

This Thesis is dedicated to my husband and our two children.

February 2007

Annette Torgunrud Kristensen

List of papers

I

Kristensen AT, Bjørheim J, Minarik M, Giercksky K-E, Ekstrøm PO: **Detection of mutations in exon 8 of TP53 by temperature gradient 96-capillary array electrophoresis.** *Biotechniques* 2002, **33:** 650-653.

II

Bjørheim J, Abrahamsen TW, Kristensen AT, Gaudernack G, Ekstrøm PO: **Approach to analysis of single nucleotide polymorphisms by automated constant denaturant capillary electrophoresis.** *Mutat Res* 2003, **526:** 75-83.

III

Kristensen AT, Bjørheim J, Wiig J, Giercksky KE, Ekstrøm PO: **DNA variants in the ATM gene are not associated with sporadic rectal cancer in a Norwegian population-based study.** *Int J Colorectal Dis* 2003.

IV

Kristensen A.T, Wiig JN, Larsen SG, Giercksky KE, Ekstrøm PO. **Association study of DNA variations in genes of the folate metabolism with rectal cancer in a Norwegian population.** Submitted January 2007.

V

Kristensen A.T, Wiig JN, Larsen SG, Giercksky KE, Ekstrøm PO. **Molecular detection (k-ras) of exfoliated tumour cells in the pelvis is a prognostic factor after resection of rectal cancer?** Submitted February 2007.

Abbreviations

5-FU	5- Flurouracil
A	Adenine
ACDCE	Automated Constant Denaturant Capillary Electrophoresis
AJCC	American Joint Comitee on Cancer
APC	Adenomatosis Polyposis Coli
AS-PCR	Allele Specific Polymerase Chain Reaction
AT	Ataxia Telangiectasia
ATM	Ataxia Telangiectasia Mutated gene
ATP	Adenosine Triphosphate
BAX	BCL2-associated X protein
BCL2	B-cell CLL/lymphoma 2
BP	Base Pair
BRAF	v-raf murine sarcoma viral oncogene homolog B1
BRCA	Breast cancer gene
C	Cytosine
CASP5	Caspase 5, Apoptosis-related Cysteine Peptidase
CBS	Cystathionine β-synthase
CDCE	Constant Denaturant Capillary Electrophoresis
CDGE	Constant Denaturant Gel Electrophoresis
CE	Capillary Electrophoresis
CHIP	Children's Hospital Informatics Program
CIN	Chromosome Instable
CRC	Colorectal Cancer
CTCE	Cycling Temperature Capillary Electrophoresis
CTNNB1	Catenin, Beta-1
DCC	Deleted in Colorectal Carcinoma
DCE	Denaturant Capillary Electrophoresis
DGGE	Denaturing Gradient Gel Electrophoresis
DHFR	Dihydrofolate Reduktase
dHPLC	denaturant High Performance Liquid Chromatography
DNA	Deoxyribonucleic acid
DPD	Dihydropyrimidine Dehydrogenase
DSBs	Double Stranded Breaks
dsDNA	double stranded DNA
dTMP	Deoxythymidine 5'-phosphate
dUMP	Deoxyuridine Monophosphate
ERK	Extracellular Signal–regulated Kinase
FAP	Familial Adenomatous Polyposis
Fas/Apo1	Integral membrane protein with killer domain
G	Guanine
GC-clamp	Guanine and Cytosine rich domain
GDP	Guanosine Diphosphate
GRB2	Growth factor receptor-bound protein 2
GTP	Guanosine Triphosphate
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
In vivo	(with)in the living organism

Killer/DR5	Death receptor 5, DR5, KILLER, KILLER/DR5
K-RAS	Kirsten RAS gene, Ki-RAS
LOH	Loss of Heterozygozity
MADH2, 4	MAD (mothers against decapentaplegic, Drosophila) homolog 2,4
MAPK	The Mitogen-Activated Protein Kinase
MB	MegaBace
MEK	Mitogen Extracellular signal–regulated Kinase
MEKK	MAP Kinase Kinase Kinase
MIN	Microsatellite Instability
MLH1	MutL, E. Coli, homolog of, 1
MS	Methionine Synthase
MSH 2,3,6	mutS homolog 2, 3 and 6
MTHFR	5,10-Methylenetetrahydrofolate Reductase (NADPH).
MTRR	Methionine Synthase Reductase
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
PIK3CA	Phosphatidylinositol 3-kinase, Catalytic, Alpha polypeptide
RAF	Serine/theronine Phosporylation Cascade
RAS	Rat Sarcoma virus
RFLP	Restriction Fragment Length Polymorphism
SAM	S-adensylmethionine
SNP	Single Nucleotide Polymorphism
SHMT	Serine Hydroxymethyltransferase
SOS	Son Of Sevenless
SSCP	Single Strand Confirmation Analysis
ssDNA	single stranded DNA
T	Thymidine
TCF4	Transcription Factor 4
TGFBR2	Transforming Growth f\Factor, Beta receptor II
TGGE	Temperature Gradient Gel Electrophoresis
THF	Tetrahydrofolate
TME	Total Mesorectal Excision
TNF beta	Transforming growth factor, beta receptor I
TNM	Tumour, Node, Metastasis staging system
TP53	Tumour protein p53
TS	Thymidylate Synthase
TTGE	Temporal Temperature Gel Electrophoresis
UV	Ultraviolet

General introduction

Colorectal cancer

Cancer develops through accumulation of several different genetic changes in the cell. The suggestion that cancer was a genetic disorder in somatic cells, was first presented in the end of the last 19th century by Hansemann [1]. Some years later in 1914 Boveri [2] proposed that chromosome abnormalities were fundamental to the development of cancer. During the last decade, numerous studies have been published, showing that several oncogenes, tumour-supressor and repair genes are important in colorectal cancer. These genes have an important impact as prognostic factors in colorectal cancer, and may also be essential to improved treatment and outcome for the patient.

Colorectal cancer is one of the most common malignant diseases in the Western countries, and increased incidence has been observed over the past decades [3]. In the Norwegian population, the Cancer Registry of Norway Institute of Population-based Cancer Research recorded 3482 new cases of colon (2345) and rectum (1137) cancer in 2004 [4]. Cancer in the colon- and rectum compose 15% of all the new cancer cases, and is the most prevalent cancer site within the Norwegian population. Rectal cancer has a male/female incidence ratio of 1.5, while colon cancer has a male/female ratio incidence of 1.1 [4]. Cancer in the colon and the rectum mostly affect the elderly and the age specific incidence of rectal cancer increases sharply after the age of 50 years [5]. Some factors contribute to the increased risk of colon and rectal cancer. Familial Adenomatous Polyposis (FAP) is one of the known autosomal dominant inherited colorectal cancer (CRC) diseases, but accounts for less than 1% of all cases. This is a disease recognised by formation of polyposis at a young age [6]. Hereditary non-polyposis colorectal cancer (HNPCC), also known as Lynch syndrome is another autosomal dominant cancer syndrome and is the most common form of hereditary colorectal cancer accounting for as much as 5 % of all CRCs [7]. HNPCC typically develops around the age of 45. Another factor causing increased risk of colon and rectum cancer is chronic inflammatory diseases such as Ulcerative colitis and Crohns disease [8]. Patients that have received pelvic radiation in association with gynaecological cancers have been observed to have an increased risk 2-3 times compared to that of a normal population [9].

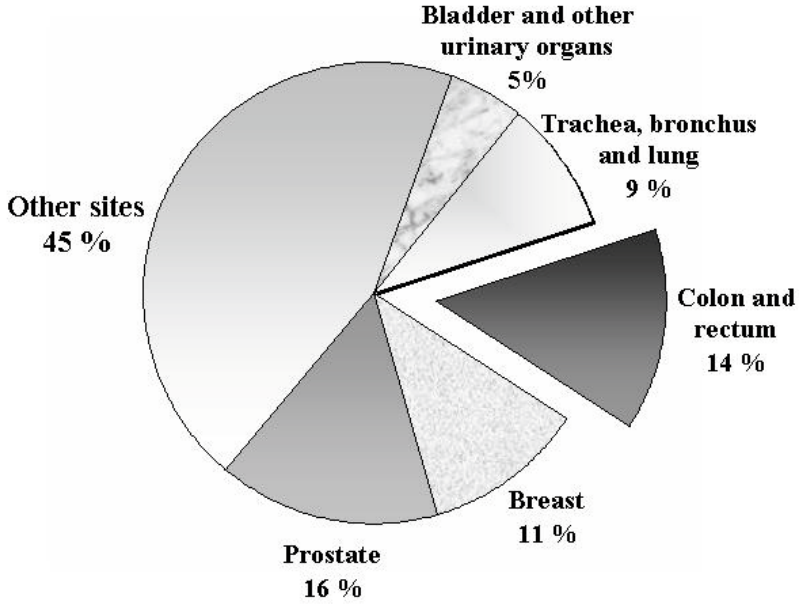

Figure 1. The distribution of cancer types in Norway (2004). This was obtained from the Norwegian Cancer Registry [4].

Treatment and outcome

The choice of treatment for colorectal cancer is dependent on the tumour stage at diagnosis. Surgery is the main and most efficient treatment for colorectal cancer and most of the patients with primary tumours and local recurrences will have their tumour removed. Early detection of colorectal cancer is correlated with high survival rates, as 90% of patients diagnosed with TNM stage 1 tumours are alive 5 years after the initial diagnosis, compared to a steady decrease in 5-year survival rates in later stages, down to 10% in stage IV patients [10].

Patients with advanced local, regional or metastatic cancer require additional therapy, consisting of chemotherapy and radiation. In Norway, 5-fluorouracil (5-FU) in combination with calsiumfolinate used to be the standard chemotherapy for this patient group. Later Oxaliplatin has been added to this first line chemotherapy.

Radiation has a major role in the treatment of rectal cancers. Radiation can be given before surgery to cause the tumour to shrink allowing easier removal, or postoperatively to decrease the risk of cancer recurrence. Tumour extension and dissemination are the most important prognostic factors. In spite of what is believed to be a curative tumour resection

many patients later suffer from metastases or tumour recurrence. Possible causes of local recurrences are believed to be; incomplete resection of the primary carcinoma [11], insufficient removal of involved regional lymphatic vessels or nodes [12], implantation of a secondary tumour near the suture line and exfoliated colorectal cancer cells released during the surgical procedure [13].

Survival from rectal cancer has been continuously improved during the past decades. The main reason for this seems to be the development of the surgical technique called total mesorectal excision (TME) [14;15]. An additional local national contributing factor was the establishment of The Rectum Cancer Registry at The Cancer Registry of Norway [4;16]. The main element in this initiative was an educational program to standardize and optimize surgery for rectal cancer at a national level. At the same time pathologists were educated in the optimal principles of handling and describing rectal cancer specimens. All surgical departments treating rectal cancer were invited to transfer their clinical data to this registry. Each department regularly receives its own results together with the national average for comparison and quality control.

Genetic model for colorectal cancer

Colorectal adenoma-carcinoma sequence model

Development of colorectal cancer occurs through a number of genetic and epigenetic changes, and was described as an adenoma–carcinoma sequence in 1975 by Muto et al. [17]. Almost twenty years later these stages were used in a genetic model put forward by Fearon and Vogelstein in 1990 [18]. This model describes the stepwise progression from normal to dysplastic epithelium to carcinoma associated with multiple genetic alterations. The authors stressed that mutations in at least four to five regions, including Adenomatous Polyposis Coli (APC), Kirsten RAS gene *(k-ras*), deleted in colorectal cancer (DCC) and Tumour protein gene p53 (TP53) were required for formation of a malignant colorectal tumour. Further studies have shown that the order of genetic events is essential [19;20]. This concept not only provides an excellent model to study the genesis of invasive cancer, but also offers a means of preventing colorectal cancer by endoscopic removal of precursor lesions.

Differences between the left- and the right side of colon

CRC's that arise proximal (right) or distal (left) are different with respect to epidemiological, clinicopathological, biochemical as well as genetic factors [21]. This lead to the suggestion that proximal and distal tumours follow broadly different molecular pathways in carcinogenesis. Two distinct pathways have been identified which result in colorectal cancer. The tumour suppressor pathway, also termed the chromosomal instability pathway (CIN), account for 85% of the colorectal carcinomas and most sporadic carcinomas. The CIN pathway is characterized by loss and gains of chromosomes (aneopleoidy) often 5q, 17p and 18q as well as loss of heterozygozity (LOH), which is a loss of one of the parental alleles present in cells, and mutations in p53. The CIN pathway most often appears in the distal part of the colon.

The second mutational pathway, the microsatelitte instability (MIN) pathway accounts for the remaining 15% of colorectal carcinomas [22]. This pathway often has a family history of colorectal cancer suggesting a genetic contribution or common environmental exposure among family members, or a combination thereof. Existence of multiple alternative genetic pathways have been suggested [23;24]. The MIN pathway is characterized by defects in the mismatch repair systems, leading to downstream mutations in genes such as TGFBR2 (transforming growth factor-β) [25], BAX (BCL2-associated X protein) [26], *k-ras* (Kirsten RAS gene), APC (adenomatosis polyposis coli) [27] and candidate target genes such as WISP-3 [28]. Tumours with MIN have some clinicopathological features in common, like proximal location in the colon [29]. MIN are also more likely to be present at an advanced age [30] and to be associated with female gender [31].

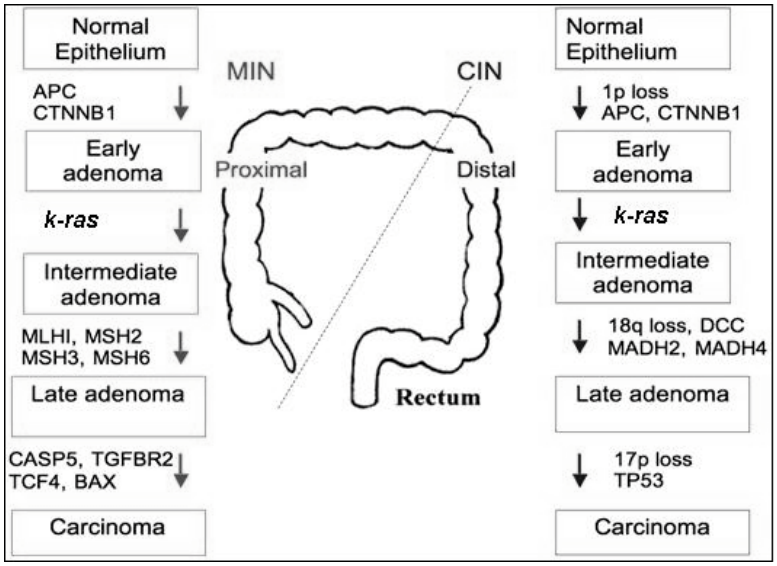

Figure 2. Genetic and epigenetic changes in the adenoma-carcinoma sequence in left versus right-sided carcinogenesis. Modified after [32-33].

Differences between rectum and colon

Colon and rectal cancers were previously considered a common entity, but are now regarded as different diseases with a number of common components. Rectal carcinomas are assumed to arise through similar mechanisms as in distal colon tumours (descending and sigmoid colon). It is therefore not surprising that significant differences in rectal and colon carcinomas with respect to age and gender of the patient have been reported [34-36]. Breivik et al [36], showed that proximal and distal colorectal cancers evolve by different genetic pathways and that these pathways are influenced by sex-related factors. Frattini et al.[37] did a study on different genetic features associated with colon and rectal carcinogenesis. The different pathways observed and their distribution were summarized as followed; the number of *k-ras* mutations detected were significantly higher in colon than rectal tumours, and a mutational pattern restricted to the APC gene was more common in rectal than in colon tumours. According to Kapiteijn et al. [34] the p53 pathway seems to be more important in rectal cancer than in colon cancer, and also reported a correlation of p53 with reduced disease free survival in rectal cancer, but not in colon cancer. It is well known that the clinical behaviour of colon cancer and rectal cancer is different. In rectal cancer local recurrences is a major problem, whereas in colon cancer distant metastasis and

carsinomatosis are the most important problems. This again suggests that the cause factors and the molecular basis may differ between colon and rectal cancer. When prognostics markers are investigated in larger series, such differences in ethiology and biological behaviour between colon and rectal cancer should be considered.

Peritoneal cavity

The peritoneum is a serous abdominal membrane, which lines both the abdominal wall and the intra-abdominal viscera. Although the sheet of body tissue ultimately forms one continuous sheet, two types or layers of peritoneum are usually referenced to as well as the potential space between them: The outer layer, called the parietal peritoneum, is attached to the abdominal wall. The inner layer, the visceral peritoneum, is more or less wrapped around the internal organs located inside the abdominal cavity. The potential space between these two layers is the peritoneal cavity [38]. The term mesentery is often used to refer to a double layer of visceral peritoneum. Blood vessels, lymphnodes and nerves are located between these layers. It should be noted that the space between these two mesenterial layers is technically outside of the peritoneal sac, and not within the peritoneal cavity see Figure 3.

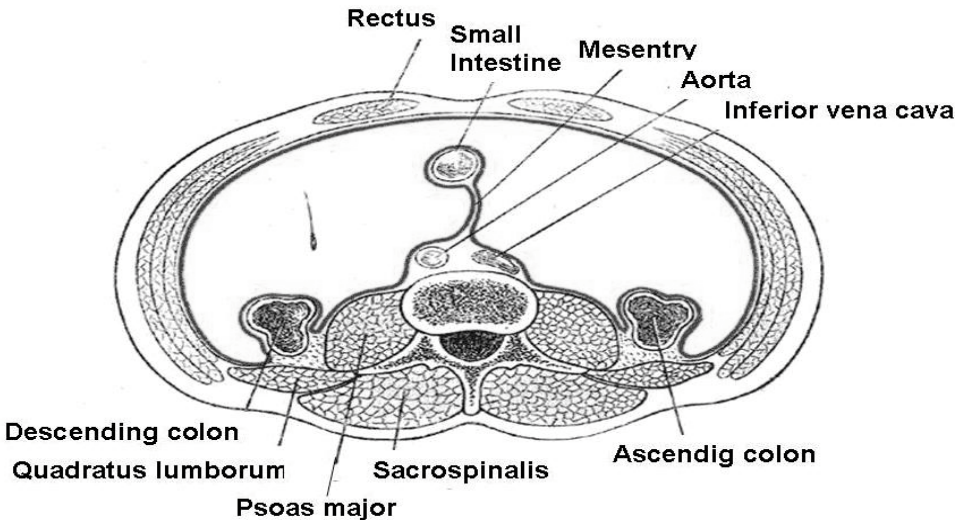

Figure 3. This picture show horizontal disposition of the peritoneum in the lower part of the abdomen. http://education.yahoo.com/reference/gray/illustrations/figure?id=1038 (This figure has a GNU Free Documentation License).

The initial dissemination of rectal cancer tumours cells occurs through three routes: lymphs, portal blood, and peritoneal surfaces. Although lymphatic and hematogenous metastases indicate an aggressive disease process, it is possible that dissemination to the peritoneal surfaces may be the result of direct contamination with cancer cells of the parietal and visceral peritoneum. This suggests that viable tumour cells with proliferative and perhaps metastatic potential had been shed from the primary tumour site either before removal of the tumour or during surgical resection [39]. Patients with overt peritoneal or local metastases from colorectal cancer have a poor prognosis [40]. However, aggressive treatments by surgery and infusion of intra-peritoneal chemotherapy have been tried and appear to benefit selected patients [41].

Histopatholgically stages

The goal of staging is to determine the extent and location of the tumour in order to develop appropriate treatment strategies. The staging of rectal cancer closely approximates the staging of colon cancer. Dukes staging was introduced in 1932 [42] to characterise the extent of tumour dissemination. This placed the cancer into one of three categories (Stages A, B, C). Later the system was modified to include a fourth stage (Stage D) [43]. Many other staging systems have been proposed trying to define a scheme which is more predictive, e.g. The Gunderson-Sosin modification of the Astler-Coller system [44] and Jass et al. [45]. More recently, the American Joint Committee on Cancer (AJCC) has introduced the TNM staging system, which places the cancer into one of four stages (Stages I-IV) [46]. Listed below in Table 1 are the Duke and TNM staging systems.

Dukes' Stages	Disease type	Stage	TNM staging		
			T	N	M
A	Local	I	T1, T2	N0	M0
B	Local	II	T3-T4	N0	M0
C	Regional	III	T1-T4	N1-N2	M0
D	Distant	IV	T1-T4	N1-N2	M1

Table 1. This table show TNM staging. T1-4; the increasing spread of primary tumour, N; Lymph node metastasis, M; distant metastasis.

In addition to the TNM staging the resection margins of the tumour are also an important part of the staging procedure. It has been shown that the distance from the tumour to the circumferential resection margin is the most powerful predictor for local recurrence and not the T stage [47;48]. The local surgical achievements are staged as follows: R0 microscopically free circumferential and distal margins, R1 microscopically involved margins, and R2 locally macroscopic residual cancer or no resection.

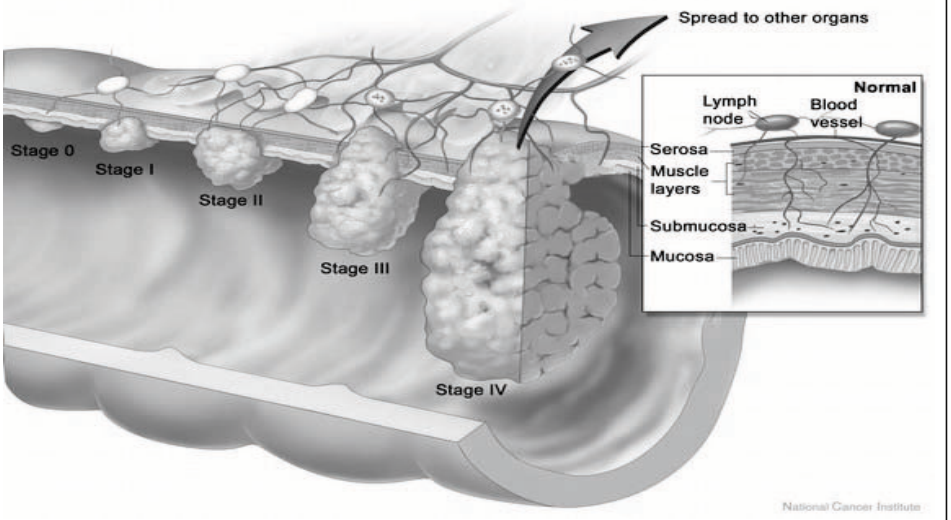

Figure 4. Shows the different TNM stages of colorectal cancer. This figure is reproduced from the National Cancer Institute http://www.cancer.gov/cancertopics/pdq/treatment/rectal/Patient/page2#Keypoint7.

Signal pathways and biological markers

Selected genes from three signalling pathways have a central role in this thesis. The first one is the Ras-pathway with the MAPK-kinase pathway. One major function of the ras protein family is to couple growth factors to the Raf-mitogen-activated protein (MAP) kinase kinase-MAP kinase signal transduction pathway, which leads to the nuclear expression of early response genes. The mutated *k-ras* product maintains a prolonged state of activation leading to cellular proliferation. Mutations in *k-ras* is described as an early event in the process of colorectal carcinogenesis [18]. Another important pathway is the TP53-BAX pathway, which is shown to be responsible for the progression of the colorectal tumour [19]. The last pathway described in this thesis is the folate metabolism. This metabolism is thought to contribute to colorectal carcinogenesis by altering both DNA methylation and nucleotide synthesis. Genes coding for metabolism enzymes, receptor proteins or protein target of chemotherapy agents often present different genetic polymorphisms able to influence drug sensitivity, toxicity and dosing [49].

Ras-pathway with the MAPK-kinase pathway

A large number of oncogenes have been identified in human tumours, including colon cancer. The *k-ras* gene is the most commonly mutated *RAS* family member in colon cancer, although *N-RAS* mutations are also observed in a small percentage of colon cancers [50]. The *RAS* family genes encode a highly conserved family of 21-kDa proteins involved in signal transduction. Ras serves as a GDP/GTP related binary switch with a crucial role in the transmission of growth regulating signals within the organism, and may be used as a critical terminal through which signals are passed on to other signalling modules (see Figure 5). The best characterized of the signalling pathways regulated by Ras is the mitogen-activated protein kinase (MAPK) pathway. This pathway is activated when Ras activates a Serine/theronine Phosporylation Cascade (Raf). Raf thereafter phosphorylates and activates a mitogen extracellular signal–regulated kinase (MEK), which phospyrates a mitogen-activated protein kinase (MAPK). This in turn activates an extracellular signal–regulated kinase (ERK) that plays pivotal roles in a wide variety of developmental cellular processes, including growth, division, and differentiation [51].

The mutated *k-ras* product maintains a prolonged state of activation leading to cellular proliferation. Novel activating mutations in sporadic CRC have recently been identified on major kinase encoding genes such as BRAF [52] and PIK3CA [53]. The presence of these

activating point mutations, including the well-characterized *k-ras* oncogene mutations, is represented in up to 75% of cases of CRC. These genes, which have been implicated in the adenoma-carcinoma transition, cause deregulation and constitutive activation of the MAP AKT/kinase pathways, rendering growth advantages to colon tumour cells [54;55]. Mutated *k-ras* may also inhibit the TP53-BAX signalling pathway by phosporylating pro-caspase-9, and thereby inhibiting cythochrome c induced apoptosis [19;51].

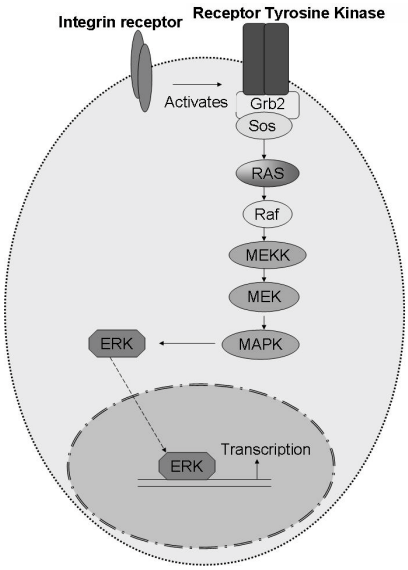

Figure 5. Modified after Juliano et al. [56]. The Integrin receptor activates the receptor tyrosin kinase. Secondly, Integrin enhances the efficiency of the cytoplasmic cascade comprising Raf, MEK and Erk. This activates adaptor proteins such as Grb2, which in turn can recruit other proteins such as SOS, a partner of Grb2. Grb2 is an exchange factor for Ras GTPase. Activation of Ras at the membrane by SOS leads to the recruitment of Raf. Raf is the first kinase in the intracytoplasmic signalling pathway leading to Erk. Raf subsequently phosphorylates and activates MEKK (MAP kinase kinase /Erk kinase) leading to activating of MEK (MAP kinase kinase) and MAPK (Map kinase), which leads to the activation of Erks. Activated Erk migrates to the nucleus, where it can phosphorylate its targets, leading eventually to cell replication.

The TP53-Bax pathway

The TP53-Bax pathway is important in DNA damage, cell survival and proliferation. TP53 gene codes for a protein that acts as a transcription factor and serves as a key regulator of the cell cycle, coupling stimuli that promote cell division to those that promote cell death [57]. Mutations in the TP53 tumour suppressor gene are found at high frequency in a wide range of human cancers [58]. TP53 is also in control of Bax, which is a pro-apoptopic member of the BLC-2 family. It reduces cytochrome c released from mitochondria resulting in apoptosis (see Figure 6).

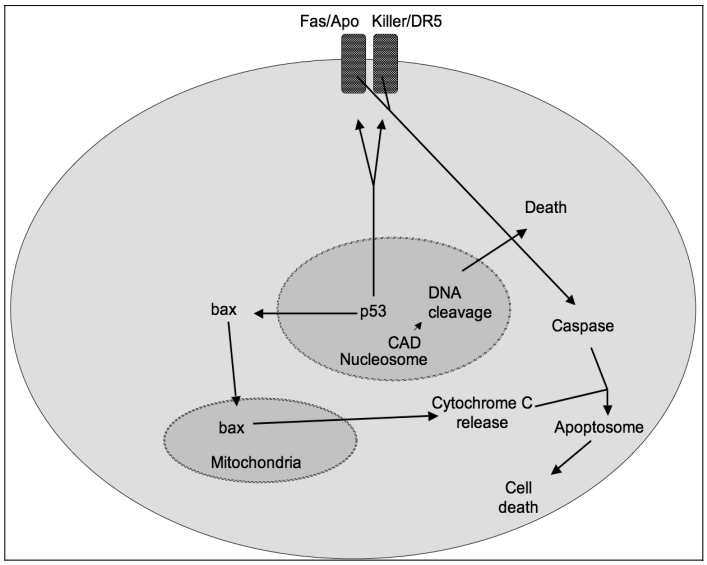

Figure 6 TP53-Bax pathway. P53 transcritionally activates Bax, and integral membrane proteins Fas/APO1 and Killer/DR5. These integral membrane proteins are death receptors and activation of these results in activation of the caspase cascade. Cythocrome C released by Bax also activates the caspase cascade, which in turn activates endonuclease and thereafter DNA cleavage. This Figure was modified from [57].

Folate metabolism

Folate is a water-soluble B vitamin. The primary function of folate is as a coenzyme in reactions that require transfer of a single carbon moiety in different oxidative states as a methenyl, methylene, or methyl group. These reactions typically involve synthesis of compounds such as thymidine, a pyrimidine base necessary for synthesis of DNA. Consequently, folate status could potentially be perturbed by polymorphisms in genes important in the folate metabolism (see Figure 7). Folate deficiency could also affect malignancy by causing DNA hypomethylation and proto-oncogenic activation by inducing uracil misincorporation during DNA synthesis, leading to catastrophic DNA repair, DNA strand breakage and to chromosome damage [49;59]. A deficiency in folate plays an important role in the pathogenesis of anemia [60], atherosclerotic cardiovascular diseases [61], neural tube defects [62] neuropsychiatric and cognitive disorders [63] and cancer [64;65]. Colon cells are subject to rapid turnover, and thus are the site of high rates of DNA synthesis. Several studies have shown a link between low folate status and indicators for DNA damage [59;66], and that dietary folate intake could reduce the risk of colon cancer [67;68].

In cancer treatment it is well known that there are inter individual differences in tumour response and normal tissue toxicity [69-72]. Many clinical variables with inter individual differences have been associated with e.g. age, gender, diet and drug-drug interactions.

However the major clinical variable is the DNA variation in genes of different populations. Genes that code for metabolism enzymes, receptor proteins or protein targets of chemotherapy agents often present different polymorphisms that can influence drug sensitivity and optimal dosing.

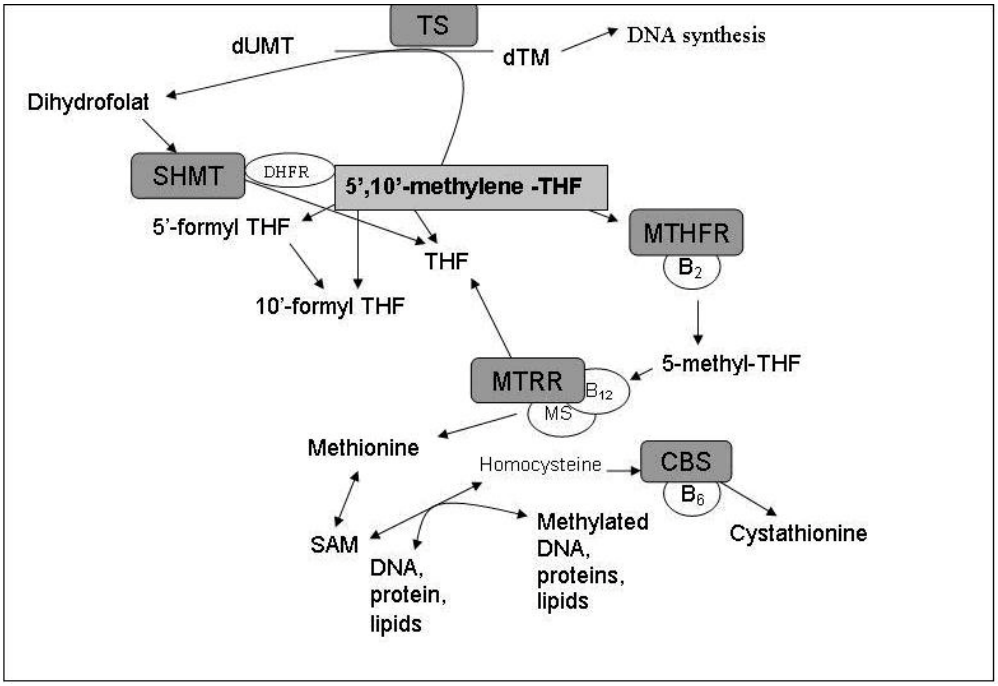

Figure 7 Highlights of the folate metabolism, including key enzymes involved and major regulatory mechanisms. Key enzymes are denoted as black boxes, substrates as white boxes. B_2, B_6 and B_{12} are vitamins.

Ataxia telangiectasia and the ATM gene

Appropriate cellular signalling responses to DNA damage and the ability to repair DNA are fundamental processes required for an organism's survival. Ataxia telangiectasia (AT) is a rare neurodegenerative disease that results from defective DNA damage signalling [73]. This disease is characterized by neurological and immunological symptoms, sensitivity to radiation, and cancer predisposition. AT patients have a higher incidence of breast cancer and leukaemia than the general population and about one-third of patients develop a clinical significant malignancy during their lifetime [74]. The incidence of AT heterozygous individuals in the general population is estimated to be 0.5–1% [75-77]. Understanding the molecular basis of AT has provided many critical insights into the cellular response to DNA

double-strand breaks (DSBs). DSBs are the major DNA lesion leading to chromosomal aberrations and reliable repair is crucial for maintaining genomic stability. The gene mutated in AT, the *ATM* gene, has a role as DSB detector. The *ATM* gene encodes a protein kinase that is mainly distributed in the nucleus of proliferating cells. It appears to play a central role in cell cycle regulation, DNA repair and apoptosis and control of cellular responses to DNA damage [78]. The *ATM* gene has been fully elucidated [79] and the complete genetic sequence is known [80]. However, it's large size (146 kb) and complex 62-exon structure has greatly complicated the process of screening large sample sets for all possible sequence variations. DNA variations in the *ATM* gene have been studied in colorectal cancer [81-83]. These studies showed that expression of *ATM* could predict survival in colorectal cancer, and increase radio sensitivity.

Methods to detect DNA variants

One of the major trends in post-genomic research is the exploration of genetic variation in humans. The variation of a single base may lead to change in cellular behaviour, and this change could serve as a diagnostic marker or a target for future therapeutic intervention [84]. The completion of the Human Genome Project in 2003, and sequencing of the entire genome in more than one individual, made it clear that there are major individual differences in the genome, especially between different ethnic populations. Worldwide efforts to collect DNA variations have lead to several public databases. Validation of DNA variations and estimation of their allele frequencies are useful, because these markers can be used in genetic studies [85]. A high-throughput capacity is crucial to complete the large studies necessary for reports of such genetic studies.

Denaturant gradient gel electrophoresis and related techniques

Many techniques are now available for discovery and scoring of DNA variations. One group of methods, the melting gel techniques have proven to be amenable and powerful tools for analysing DNA variations. The reason for melting gel techniques popularity rests upon the well-documented theoretical foundation of these methods, and the few laboratory steps that are needed in the analysis in combination with high specificity and sensitivity. Denaturant Gradient Gel Electrophoresis (DGGE) has been the most frequently used melting gel electrophoresis technique and was first described by Fischer and Lerman [86]. DGGE detects mutations (small deletions and insertions, point mutations) by separating PCR

amplified DNA fragments, which differ from wild-type DNA in their melting behaviour, on a denaturing gradient gel. Fragments analyzed by DGGE must have appropriate melting characteristics, namely a low temperature melting domain and a single contiguous high temperature melting domain. A melting domain is defined as a region of DNA that melts cooperatively at approximately the same temperature or concentration of denaturant. The melting characteristics of a fragment can be predicted from its primary sequence, and computer algorithms are available to perform this analysis [87]. To include a larger part of the genome (those without a natural high temperature domains) applicable to this method Myers et al. [88] has reported that a GC rich 40-basepair region attached to one of the primers during initial PCR amplification increases the number of fragments that can be analysed with melting gel techniques. Thus constructing a high melting domain adjacent to the target sequence of the fragment. However there are some fragments with a single base alteration where no thermodynamic difference between the wild-type homoduplex and mutant homoduplex is achieved, even though a GC- rich content is attached [89]. These fragments will not separate the homoduplexes. However heteroduplexes in the low temperature domain of a fragment will resolve from the corresponding homoduplexes, so that the mutation fraction can be seen. To determine the nature of the variant DNA sequence, the heteroduplex peak can be collected and subsequently sequenced [90;91].

DGGE employs a linear gradient of denaturant in a polyacrylamide gel. Double-stranded DNA molecules migrate into the gradient in the gel until the point where a discrete denaturing concentration is reached. This results in a partially melted, Y-shaped molecule where mobility through the gel will decrease drastically. The separated amplification products can be made visible on an UV-transilluminator after incubating the gel with an ethidium bromide solution, cyber green or other DNA intercalating dyes. Separation in the gel can be seen in Figure 8A. In this figure we can observe 4 bands. During the PCR amplification of a fragment containing a point mutation four different variations of the amplification product will be produced. Two homoduplexes, representing the wild-type fragment and the mutant fragment with perfectly matched sequence. Secondly, two heteroduplexes, representing imperfectly match of forward wildtype and reverse mutant sequence or forward mutant fragment and reverse wildtype.

There have been modifications in melting gel techniques. Temporal Temperature Gel Electrophoresis (TTGE) [92;93] is applicable without the requirement of using a chemical denaturering gradient. The temperature is increased gradually, that result in a linear temperature gradient over the timecourse of the electrophoretic run. Temperature Gradient

Gel Electrophoresis (TGGE) [94] provides a temperature gradient instead of a chemical gradient.

Constant Denaturant Gel Electrophoresis (CDGE) [95] is another modification where the method has the advantage of enabling the fragments to migrate at a consistently different rate through the whole gel. This allows greater separation between mutant and wild-type fragments [95-97].

Modification of the CDGE to the capillary platform was first published by Khrapko in 1994 [98;99] (see Figure 8B). The application of Constant Denaturant Capillary Electrophoresis (CDCE) was first developed on a laboratory-assembled instrument [100]. Benefits of capillary-based electrophoresis (CE) include fast sample runs and data storage capability and increased limit of detection; in addition, only minimal amounts of sample are required with CE techniques. Elevating the temperature of a section of the capillary change the denaturing conditions for CDCE. Various methods have been used to apply temperature gradients to capillaries including the use of a voltage ramp [98], an external heating plate [101], Peltier-based control of surrounding liquid [102] and heated air [103]. A further improvement of CDCE allowing two-point detection and automated fraction collection has been reported [104;105]. Conversion of CDCE into regular commercial capillary DNA sequencing instruments has resulted in automated and standardized protocols [106]. Virtually any commercial single or multi capillary instrument may be used. The automation allows for rapid analysis of a large number of samples for a short period of time, with no interference by the operator. The method was first described with the use of an ABI 310 Genetic Analyzer, in which up to 48 samples could be analysed without need of the operator [107]. This method has also been adapted to the Megabace™1000, allowing for analysis of 96 samples within 40 minutes [108;109], ABI 3100 genetic analyzer [110], a SCE2410 24-capillary instrument from Spextrumedix [103;105], Beckman Coulter eight capillary (unpublished data) and tested on MegaBACE 4000 with 384 capillaries (unpublished data).

The temperature control for the method has been improved by modifying the CE system with the use of a temperature gradient [109].

Samples are analyzed with a temperature gradient starting above and declining beneath the optimal separation temperature, controlled by the computer software. This modification compensates for the temperature differences in the capillary chamber resulting in a robust method. Additional improvement of the gradient is accomplished by cycling the gradient several times around the optimal separation temperature [111;112].

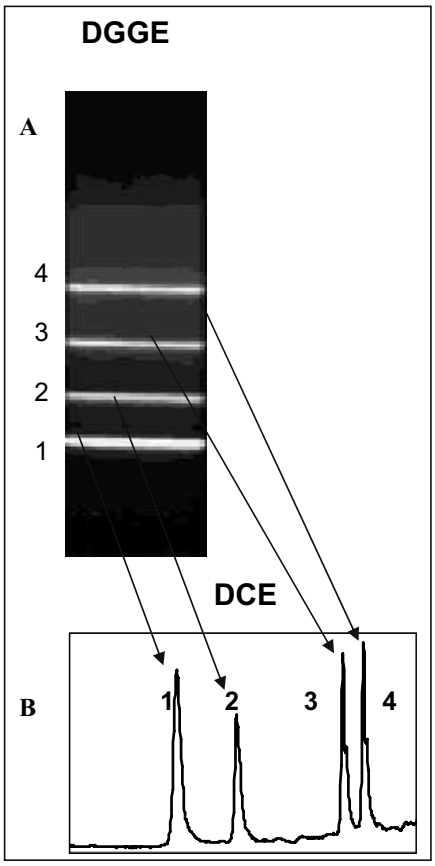

Figure 8. This figure shows the adaption of DGGE (A) to Denaturant Capillary Electrophoresis (DCE) (B), where the picture of a gel with 4 bands (nr 4 and 3 heteroduplexes 2; mutant and 1; wild type) corresponds to the electropherogram obtained by DCE.

Application of Denaturant capillary Electrophoresis (DCE)

Mutation analysis

It is widely accepted that human cancer is a genetic disorder caused by sequential accumulation of mutations in oncogenes and tumour suppressor genes. These tumour-specific mutations in cellular processes underlying tumorigenesis have proven to be useful for diagnostic and therapeutic purposes**.** In the past, the selection of genes chosen for mutational analyses in cancer has been guided by information of known functional attributes of individual genes or gene families. With the determination of the human genome sequence it is now in principle possible to examine the cancer cell genome in a comprehensive and unbiased manner. Such an approach not only provides the means to discover other genes that

contribute to tumorigenesis, but can also lead to mechanistic insights that are only evident through a systems biological perspective. One consideration of mutation detection analysis is the possibility of detecting mutations in a small fraction of samples. To study rare variants in the human population or new somatic or germline mutations, a technique is required which can detect and separate any and all of the variety of mutations in a target gene at fractions from the wild-type DNA. This can be important for early detection of malignant diseases, detection of remaining cells after surgery and possibly for prognosis and outcome of the disease. Several different mutation analyses have been performed by DCE [90;92;100;104;105;113-119]. A major improvement to the method was conversion to commercially automated capillary instruments. The first report was on a single capillary instrument; the ABI 310 Genetic Analyzer, where mutations of *k-ras* exon 1 [120] and exon 5-8 in TP53 gene were analyzed [107]. The same mutation analyses have also been performed on the commercial MegaBACE™1000 instrument, [108;109;111;121;122], and a SCE2410 from Spextrumedix [103;105].

Single Nucleotide Polymorphism (SNP)

Single nucleotide polymorphisms or SNPs are DNA sequence variations that occur when a single nucleotide (A, T, C or G) in the genome sequence is altered. For a variation to be considered a SNP, it must occur in at least 1% of the population. SNPs, which make up about 90% of all human genetic variation, occur on average once every 87 bp along the 3-billion-base human genome. 8 354 954 SNPs are currently registrated in the CHIP Bioinformatic tool database [123;124], however only 60 % are validated, 11% of these SNPs have frequency data and 40 % of the SNPs are located in genes. Two of every three SNPs involve the replacement of cytosine (C) with thymine (T). SNPs can occur in both coding and noncoding regions of the genome. Although more than 99% of the human DNA sequence is identical in all human beings, variations in DNA sequence can have a major impact on how humans respond to disease, drugs and other therapies. This makes SNPs of great value in biomedical research and in developing pharmaceutical products or medical diagnostics. SNPs are also evolutionarily stable. This makes SNPs a popular tool to discriminate between alleles or haplotypes in population studies. The DCE method has proven to be a robust method for genotyping alleles and also detection of new SNPs. Target sequences require only PCR amplification followed by allele separation by capillary electrophoresis. The genotypes of the individuals SNPs are scored based on co-migration to

an internal standard. Due to the presence of an internal standard in all the electrophoretic runs, the specificity of the SNP analysis is increased as allelic separation of the standard is required before genotypes can be determined [89;125] (see Figure 9). Discovery of new unpublished SNPs in the target fragment are also possible with this method. Detection of unpublished SNPs will be discovered by aberrant peak pattern compared to the internal standard, and may be verified by DNA sequencing. In a study by Lorentzen et al. [126] six microsatelittes and three SNPs in two Norwegian populations were analysed by three different methods; PCR-RFLP, TaqMan® analysis and DCE. The genotype error rates when comparing PCR-RFLP with TaqMan® data or the DCE were notably higher than comparison of genotype error rate for TaqMan® and DCE. These analyses showed that both DCE and TaqMan® were superior to the PCR-RFLP method when it comes to accurate genotyping of the selected SNPs.

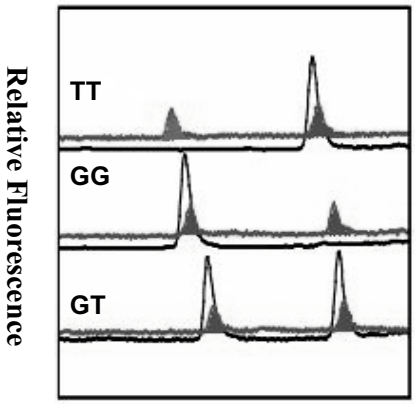

Figure 9 shows a SNP in MTHFR NCBI (rs 1801131) with three genotypes (thin) co-eluted with an internal standard (thick). Heteroduplexes are not shown.

Microhaplotypes

Microhaplotypes are in general defined as the presence of several SNPs within a short DNA fragment (100-1000bp) [127]. The theoretical number of possible alleles is defined as 2^n, where n is the number of SNPs in the sequence analyzed. Possible combinations of genotypes are given by the sum of numbers from zero up to numbers of alleles. Few methods are able to analyze microhaplotypes, and the protocols are in general labour intensive and not easily automated [127;128]. Consequently, DCE has been optimized for microhaplotyping by use of the standard method. This method has been used to identify three SNPs within the ATM gene based on theoretical thermodynamics and migration of

various fragments [125]. Another study has also used this method to detect 3 microhaplotypes in the CTLA4 gene [126]. A review by Szantai et al. [129] summarizes the recent examples of novel and emerging haplotype techniques by capillary electrophoresis based on DNA fragment analysis, with DCE as one of the methods mentioned.

Gene copy number

Pooling designs are used in screening experiments in molecular biology. In some applications, the property to be screened is defined on subsets of items, instead of on individual items. Pooled genotyping is a powerful and efficient tool for high throughput association analysis, both case-control and family based [130]. The use of a pooling design may reduce the consumables and labour cost of a study compared with genotyping individuals and counting alleles. Pooling allows a far smaller number of PCR reactions and genotype assays than are used when genotyping individuals. The most important consideration with DNA pooling is to ensure that the individuals that make up a pool contribute equal amounts of DNA, from which robust PCR results can be obtained. However random experimental errors in the constitution of DNA pools and in the measurement of allele frequencies from pooled DNA should be taken into account in statistical analysis [131]. Differential amplification occurs for many SNPs and this bias should also be corrected for in the estimate of allele frequencies from pooled DNA. Several reports have stated that multiple genotyping techniques are suitable for pooled genotyping [132;133]. Pooled DNA has been used in studies of various diseases, such as head and neck carcinogenesis [134], breast cancer [135], Liddle syndrome [136] and rheumatoid arthritis [137]. The DCE method has also been shown to be an effective and cost- beneficial method for single and pooled samples [110;119;138]. There is no need for any correction of the signal of the separated alleles because both alleles are separated with the same flourophore and because fragments of the same length with a difference of one base pair will pass the detector with the same velocity [90]. By measuring the area under the peaks (alleles), quantitative information about DNA copies entering the PCR reaction are obtained. Harbo et al. [139] have recently published a study where four sets of 1000 blood donors were pooled and analyzed for 41 SNPs involved in T cell signalling. The authors also concluded that screening of SNPs in DNA pools proved to be efficient and cost-effective, because many of the reported non-synonymous, polymorphic SNPs were in fact not polymorphic in the large Norwegian cohort. Morgenthaler and Thilly [140] have recently described a

strategy to discover genes that carry multi-allelic or mono-allelic risk for common diseases with a cohort allelic statistical sums test called CAST. The techniqual approach in this paper is the DCE method. Based on genetics, technology and statistics, case cohort samples of 10,000 persons for each of 100 common diseases are proposed and evaluated.

Allelic imbalance

Most human cancers show genetic instabilities leading to allelic imbalances. Allelic imbalance may be described by means of the two-hit model by Knudson in which one allele is mutated and the other allele is lost through a number of possible mechanisms, resulting in the loss of heterozygosity (LOH) at multiple loci [141]. Allelic imbalance has been detected by allotyping using restriction fragment length or microsatelitte markers [142]. To determine this allelic imbalance (allelic loss/reduction or gain) in tumour samples, microsatelitte markers at gene loci of interest have been used. A disadvantage of using microsatelittes is that they are rare compared to SNPs and generally located in non-coding regions of the genome. To circumvent the use of microsatelittes we can apply regular SNP analysis by DCE as means for determining allelic imbalance (see Figure 10).

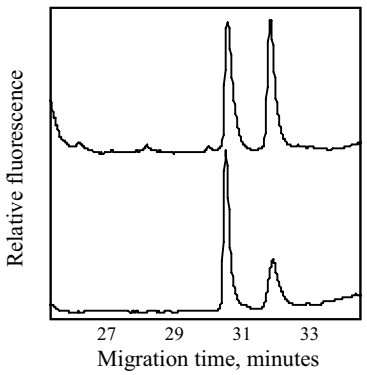

Figure 10. Allelic imbalance analyzed by DCE. Ratio of the two alleles in the upper sample is 1:1 as expected for a heterozygous sample. In the lower electropherogram a clear imbalance between alleles can be seen.

In 1974 Poland proposed an algorithm able to calculate the melting probability of thousands of nucleotides in dsDNA [143]. Based on nearest neighbour correlation in specific sequence macromolecules and basic principles of DNA thermodynamics, the algorithm stated that dsDNA melts to single stranded DNA (ssDNA) when exposed to sufficiently high temperatures and/or chemical denaturants (i.e., formamide and urea). Specifically, the length of the DNA fragment and the nucleotide sequence within the fragment defines the

melting temperature at which each bp of a DNA duplex is in perfect equilibrium between the denatured and helical state [86]. Because GC pairs consist of three hydrogen bonds, while AT pairs only have two, the temperature at which a particular DNA molecule denaturates usually will increase with higher percentage of GC pairs. Importantly, DNA variants differing by only one base will reveal different melting profiles based on the sequence variation (see Figure 11).

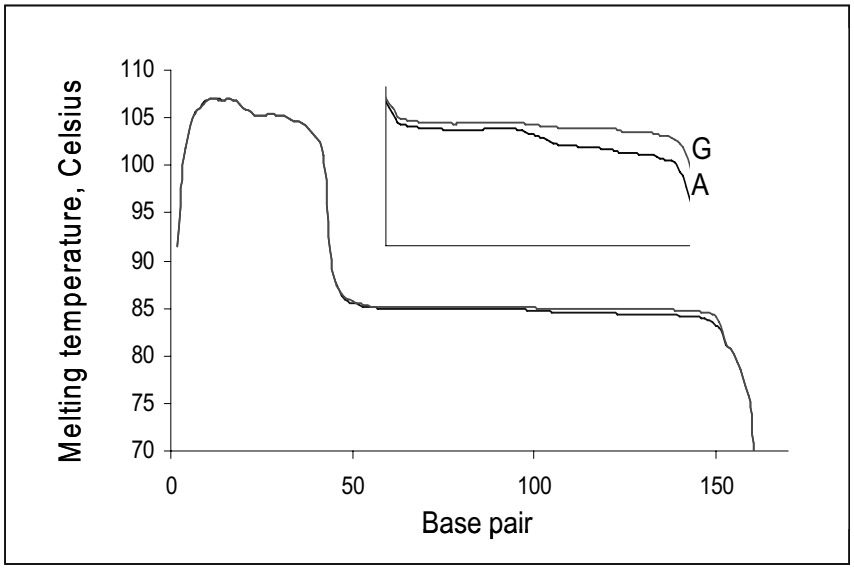

Figure 11. Thermodynamics of the DNA sequence from the gene MTHFR (NCBI rs 2274976) with use of Poland's algorithm [87].

Other scientists have subsequently proposed approximations and modifications of Poland's algorithm [87]. The need for approximations relied on the fact that an exact algorithm required computer time proportional to N^2, were N is number of bp in the target dsDNA. Computer software programs such as SQHTX (Lerman and Silverstein), Melt87 [87], WinMelt (Medprobe, Oslo, Norway) and the Poland internet web site [144] are important tools to mimic the Tm melt-curves.

Sensitivity

The quantitative sensitivity is here defined as the detection limit i.e. the lowest level an aberrant fragment sequence can be detected in a background of wild-type fragment. On the capillary platform this limit is reported to be 1% for the homoduplexes and 0.1% for the heteroduplexes for a range of target sequences [104;109;145]. Furthermore by utilizing the DCE method in combination with enrichment of mutants by fraction collection and high fidelity PCR, mutant alleles in a ratio equal to or greater than 2 x 10^{-6} have been detected [146].

Specificity

The specificity of melting gel techniques is defined as the ability to detect a wild type sample as neither mutant nor aberrant. If a PCR is not optimal it can make artefact PCR products, for example primer dimers or pseudogenes, which may form bands and peaks that lower the specificity of the test. To increase the specificity several approaches can be taken. The analysed sample with the corresponding homo and heteroduplexes can be reanalysed at slightly different denaturing conditions. In the reanalysis target sequences will migrate relative to each other due to altered denaturing conditions. Peaks that represent artifact products will not.

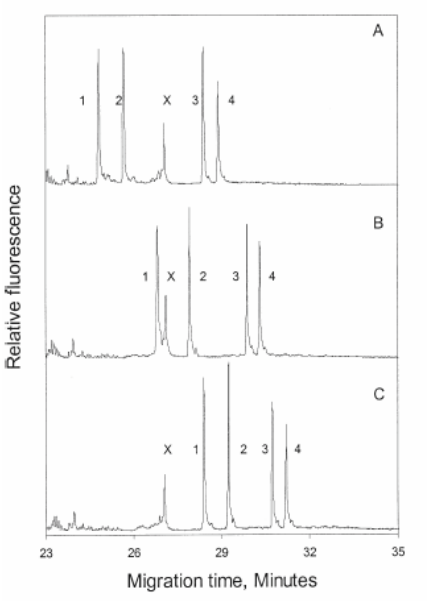

Figure 12. A sample SNP analyzed with DCE at three different cycling temperature conditions: The peaks in the electropherograms numbered from 1 to 4, represent the peaks of the sample. Peak X represents an artifact PCR product. When the temperature is increased, the average velocity of the homo and hetero duplexes is reduced, thus the amplified DNA will elute later. The artifact peak does not migrate with a different velocity when the temperature is changed. It either represents a dsDNA molecule with a different sequence or ssDNA. With permission from [147].

Another way to identify a “false positive” is to use a DCE unit with two detection points on the same capillary. This permits the precise calculation of the fragment velocity after separation in the heated zone because at room temperature all DNA fragments of the same length have the same velocity. Also the two-point detection system allows rapid distinction between double-stranded and single-stranded DNA fragments of the same length [90]. High reproducibility is important and allows direct identification of known DNA variants based on relative migration to an internal standard [89;125] see Figure 13. Notice that the electropherograms are not corrected for differences in running conditions due to temperature differences in the different capillaries.

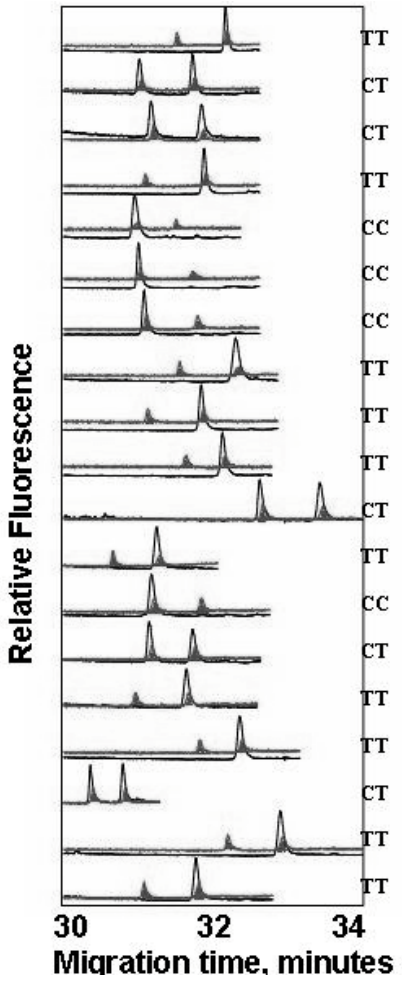

Figure 13. This figure demonstrates genotype identification of a SNP in MTHFR by co-elution with an internal standard (thick line), samples (thin line) were scored as TT, GG and GT respectively. Heteroduplexes are not shown. The figure shows representative electropherograms from different arrays within the MegaBACE instrument. The electropherograms are not corrected for differences in running conditions. The analysis time for 96 samples was approximately 34 min.

Methodological considerations

Several methodical considerations have to be taken into account before adopting this method into the lab. First of all it is important to choose genetic markers in the study design. A selection of markers is often based on previous observation of gene mutations/alterations in human cancers. Another consideration is proper primer design. When the DNA sequence of the template is known it should be possible to select target specific primers based on standard primers selection criteria's [148]. Computer programs such as Primer 3 [149] can be used to assist primer design. There is also important to make sure that primers do not anneal to pseudogenes in the template. Pseudogene is a sequence of DNA that is very similar to the normal gene but has been altered slightly so that it is not expressed. A nested PCR protocol can circumvent amplification of a pseudogene.

When designing primers it is also important to be aware of duplicate gene nomenclature given to the same gene product, distinct names given to splice variants of the same gene and alterations of number of exons and introns in a gene. Furthermore it is important to evaluate the target sequence with the allele differences with regard to the melting properties of the DNA prior to any laboratory intervention. The simulation of the melting properties by a computer program gives information if the fragments can be analysed by the DCE method. All target sequences in this thesis were analyzed with the WinMelt (Medprobe, Oslo, Norway) computer program, which revealed the theoretical melting profiles of the fragments.

Once the primers are made the main attention is to make a reliable PCR product. The PCR product is the key to this capillary method. Optimising the chemical conditions and PCR programs for the target fragments is important. One challenge is the PCR specificity and the accumulation of non-specific PCR products. We have observed labelled ss DNA as an artifact peak, which can migrate similarly to the mutant peak and the heteroduplexs peaks, and confuse the scoring of the alleles. Adding more of the unlabelled primer making an unbalance in favour of the unmarked primer can solve this problem, because the amplifying of this unlabelled ss DNA will not be visualized in the analysis. Another requirement is to make a fragment specific internal standard for the genotyping of the analyzed fragments. The internal standard is made from a heterozygous sample from the target sequence, by reamplifying a diluted sample with a different fluorophore attached to a 20mer primer with

the same sequence as the 20 last base pair of the GC-clamp. Verification of the alleles in the internal standards is done by DNA sequencing.

In this thesis we analysed tumour from rectal cancer patients. Solid tumour samples in cancer research are invaluable, as they directly reflect the *in vivo* situation. However, as soon as the tumour is removed from the body, the tumour environment is exposed to possible degradation of the sample. For that reason the surgeons placed the tissue sample directly in a tube with RNA*later*®. RNA*later*® (Ambion, United Kingdom) is an aqueous, nontoxic, tissue storage reagent that rapidly permits most tissues to stabilize and protect DNA and the more unstable RNA in fresh specimens.

Several considerations regarding the pelvic lavage fluid that has to be taken into account before interpreting the data from the analysis. The first is the viability of the disseminated cells in the lavage fluid. We concede that there are no causal connection between mutant positive DNA and viable cells in the lavage. However, at the present there are dew methods, to reliably test the viability of the tumour cells. Secondly, the surgical procedure involves a washing step with sterile water that may compromise an unknown amount of the free floating cells. However, it has previously been demonstrated that complete cell lysis with sterile water requires considerably longer period of incubation than is currently practiced [150].

Another important consideration is to secure that the detection limit for the mutation fraction is good enough, especially for the lavage fluid. Minor or moderate bleeding in the surgical area results in more white blood cells in the lavage samples, which can make mutant fraction below the detection limit. This could have been the problem in the second of the two samples because the haemostasis was not complete when lavage was carried out. It was therefore important in the first study in this thesis to prove that the detection limit was sufficient in automated standards sequencing instruments.

For many diseases such as rectal cancer, population-based studies of unrelated individuals such as case-control and cohort studies serve as standard designs for genetic association analysis and can be the most practical and powerful approach. However, extensive debate has arisen about optimum study design, and concern has been expressed about the population stratification of such approaches, which can lead to biased or false results. Unfortunately, allele frequencies are known to vary widely within and between populations, irrespective of disease status. This difference in frequencies arises because each population has a unique genetic and social history. These differences are widespread throughout the genome including many genes of known medical relevance. In effect, nearly all populations

are confounded by genetic admixture at some level. The challenge is not only to show that it exists, but also to avoid making incorrect conclusions because of it.

Aims of this project

1. To adapt DCE methods for use with commercially available single-and multi-capillary instruments.

2. To collect tissue and lavage fluid from rectal cancer patients operated at the Norwegian Radium Hospital. The samples were prospectively registrated and anonymized before being stored.

3. To apply the DCE method to detect selected DNA variations important in the adenoma-carcinoma sequence and in pharmacogenomic aspects of rectal cancer patients.

4. To study the possibility of using the developed DCE method with a marker for the *k-ras* gene to verify free tumour cells in the abdomen after surgery in rectal cancer patients.

Results in brief

Paper I

In this paper we analysed PCR products from *TP53* with temperature gradient 96-array capillary electrophoresis to evaluate the sensitivity, robustness, and throughput of the method. By gradually decreasing the temperature in combination with a chemical denaturant in the gel, separation between homoduplexes and heteroduplexes in mutated TP53 exon 8 was achieved. PCR products from a mutated sample were analysed in all 96 capillaries simultaneously. A sensitivity experiment was done, where mutants and wild-type PCR products were mixed in various ratios. The results showed a loss of heteroduplex signal below mutant fraction of 4×10^{-3}. Another feature of this study was to show the robustness of this method with respect to the separation of different mutations occurring in exon 8 of the *TP53 gene*. The mutations displayed are sited within 48 bp of the conservative region of the gene.

Paper II

The aim of this study was to demonstrate the use of automated constant denaturant capillary electrophoresis (ACDCE) of various target sequences on an ABI 310. First the target sequences were theoretically evaluated to see if they were suitable for the melting gel analysis. Primers were ordered, PCR optimised and analysed with ACDCE. To determine the best separating conditions, samples were reanalysed with increasing temperatures based on the theoretical thermodynamics of the fragments. It was possible to adjust the temperature so that the separation was achieved between both alleles for all amplified fragments. For direct identification of SNPs, an internal standard with a known genotype was added to each tube prior to ACDCE, and the sample was compared to the peak pattern of the internal standard.

Paper III

In this paper three polymorphisms in the ATM gene IVS38-8T/C, 5557 G/A and 5558 A/T were analysed in 3526 samples from different blood donors and 151 sporadic rectal cancer

patients by the method CTCE. The different alleles were resolved by melting of double strand DNA. Partial melting of DNA fragments were detected as changes in mobility during electrophoresis. All samples were run with an internal standard. Knowledge of melting behaviour for different microhaplotypes relative to the wild type combination combined with DNA sequencing allowed direct determination of microhaplotypes, and more than 7000 alleles were analysed this way. The ATM polymorphisms and microhaplotypes examined did not significantly differ between sporadic rectal cancer patients and the normal population.

Paper IV

In this study 333 patients diagnosed with rectal cancer and a control population of 384 anonymous blood donors were genotyped for nine SNPs in five different genes involved in folate metabolism. SNPs in MTHFR c.1793C>A, MTHFR c.1298A>C, MTHFR c.677C>T, MTRR c.66A>G, CBS c.699C>T, SHMT c.1420C>T, DPYD c.85G>A, DPYD c.1896G>A and DPYD IVS14+1 G>A where genotyped by cycling temperature capillary electrophoresis (CTCE). SNPs in the MTHFR gene (c.1298A>C and c.677C>T) and DPYD c.1896 A>G showed evidence of being associated with rectal cancer with p value of 0.02, 0.03 and 0.01, respectively. However by applying the stringent multiple testing corrections (Bonferroni adjustment), these p values were not considered significant in a multiple testing settings.

Paper V

In this pilot study we found the frequency of *k-ras* mutations in rectal tumours to be 30% in the Norwegian rectal cancer population and one-third of these showed *k-ras* mutations in the lavage fluid. Of the eleven possible risk factors tested with regard to patients with positive and negative *k-ras* markers in the lavage fluid only N- and R-stage were significantly different between the two groups (p=0.03 and p= 0.002, respectively). This was mainly due to a higher percentage of N0 and R0 stages in the *k-ras* negative group. Our results showed that 12 of the 19 positive K-ras were R0, which was an unpredicted result. Of the 19 patients that had a positive *k-ras* in the lavage fluid taken at the end of the surgical procedure, 13 had a primary tumour and 6 had a recurrent tumour. For the patients negative for *k-ras* mutations 178 had a primary tumour and 42 had a recurrent tumour. Survival rate was

estimated by Kaplan-meier's plot and a log rank test. The observation time was a median of 22 months. Results showed that patients positive for the lavage marker has a mean survival of 22 months compared to 46 months for where negative (p=0.006) patients. As a result, differentiation between patients with or without free tumour cells in the peritoneal cavity could indicate that an additional local or general treatment of patients with *k-ras* mutant positive lavage was necessary, independent of the R stage or other pathological features, which are the usual indicators of such treatment. This should be further studied in a large multicenter study and if possible with more mutation markers to cover a wider number of malignant tumors.

Discussion

CRC provides a good model both for the study of disease susceptibility and for the somatic evolution of epithelial cancer, due to the fact that CRC of all stages are more readily accessible than most other carcinomas. Furthermore, many of the genetic and molecular alterations, which lead to CRC, have been identified. However, both clinical and laboratory factors need to be considered when evaluating molecular tools of prognosis and prediction.

Biological considerations

In this thesis there have been two study populations. The case population were the rectal cancer patients (paper I, III, IV and V) and the control population were blood donors at the Blood Bank, Ullevål University Hospital (paper II, III and IV).

Tumour biopsies were taken with a chonchotome through a proctoscope before or in a few cases after irradiation. In some patients a tumour sample was taken from the operative specimen. One important consideration is to secure that the tissues really are tumour and not normal surrounding tissue. Most tumours show genetic instability with loss of 50/50 heterozygous distribution. Hence detection of such allelic imbalance is a good indication that a sample DNA is derived from tumour cells. In this thesis randomly selected tissue samples were subjected to analysis of allelic imbalance to validate that the sample contained tumour cells (data not shown).

We analysed rectal cancers, which have been proven in many aspects to be different from colon cancers. This may explain some conflicting results in paper III-V compared to other studies. It is well known that clinical behaviour is different between colon and rectal cancer, and it is therefore reasonable to suggest that the cause factors and the molecular basis may also differ. The colon cancer carcinomas that arise proximal (right) or distal (left) have differences related to epidemiological, clinicopathological, biochemical as well as genetic factors. Rectal carcinomas are assumed to arise through similar mechanisms as distal colon tumours (descending and sigmoid colon). Other scientists [151] recommend not to subdivide patients into colon and rectal cancer, but to see them as a homogenous component. This may explain some of the different results obtained in our study compared to others using a mixture of colon and rectal cancers. Another consideration in this thesis is that The Norwegian Radium Hospital is a third line cancer center with a high-volume of locally advanced or locally recurrent surgical rectal cancers (TNM stage II and higher).

Many markers are associated with rectal cancer, and in paper III we speculated that *ATM* could be a candidate for a modifier gene. The ATM gene was investigated, due to a target sequence with three polymorphisms (IVS 38-8 T/C in intron 38, 5557 G/A and 5558 A/T in exon 39) to demonstrate the ability of the method to detect 3 different polymorphisms in a short target sequence. This resulted in eight possible microhaplotypes at the DNA level. Furthermore, the two exonic SNPs are sited next to each other, allowing four possible amino acids in the same codon. No association was found between the polymorphisms and the haplotypes in the ATM gene with respect to the Norwegian sporadic rectal cancer patients. A number of studies have been performed to determine if there is an association between ATM mutations and cancer, but with conflicting results [81;82;152;153]. However, it is common that studies of allele frequencies vary in different populations. This can be explained by the fact that differences in population are influenced by the contribution of several genetic polymorphisms and by various environmental factors. In these cases any one of the genetic polymorphisms is neither necessary nor sufficient for the development of a given phenotype. Thus replication of association studies in different ethnic groups is of considerable importance, to better reveal the role of a given genetic polymorphism.

Polymorphisms in genes involved in the metabolism of folate and methyl groups have been implicated with risk of colorectal cancer. In paper IV we compared differences in DNA variations in genes important in the folate metabolism between rectal cancer patients and the general population. Our results indicate that there is a trend towards an association of DNA variants in genes in the folate metabolic pathway and the Norwegian population of rectal cancer patients. Different studies of these DNA variations report conflicting results [154]. Theses variations may rest upon several different factors; variant specificities of different screening technologies used, the sample size of the studies and ethnical population differences.

In the association studies (paper III and IV) we have used anonymous blood donors from the Oslo blood bank as the normal population. Hence information on the age and gender distributions was unknown. However two studies have reported on the age of the donors by surveys of people donating blood to the Oslo blood bank in 1998 and 2000, the same years these samples were collected [155;156]. They concluded that the donors ranged from 19-70 years (mean 40.5 years), and that donors were slightly overrepresented in the age 26-55. A recent study of Hinselwood et al [110] on gender determination on the same anonymous blood samples agreed with the results presented in the surveys. Rectal cancer is

predominantly a disease of the elderly. At the time of diagnosis more than 40% of rectal cancer patients in Norway are older than 75 years [4]. The rectal patient population in this thesis had a mean age of 65 years. We then have to assume that most of the blood donors are in a different age group than the rectal patient group used in these studies, and some of the people in the control group may obtain rectal cancer in a later period of their life. Associations between genotype and outcome may be confused by unrecognized population stratification, in this study the stratification is age. However the 25 years of age difference in the two populations is not significant different due to probability of death at age [157]. For this reason we can assume that no significant amounts of alleles important in the disease of rectal cancer disappear between the age of 40 and 65. To control for hidden population stratification in genetic-association studies, we are aware of proposed statistical methods using marker genotype data to infer population [158-160]. However these methods are not used in this study.

In paper IV we have corrected for multiple testing, by the Bonferroni method that allows many comparison statements to be made or confidence intervals to be constructed while still assuring that an overall confidence coefficient is maintained [161]. Results from different association studies may differ as a result of Bonferroni correction or the absence of it.

In paper V, we use *k-ras* as a tumour-associated marker to determine the presence of disseminated tumour cells in peritoneal lavage samples from patients undergoing surgery for rectal cancer. The protooncogene *k-ras* was chosen because this gene is frequently mutated in colorectal carcinoma [151;162] and located in a small hotspot region, which makes it possible to detect 80-90% of *k-ras* gene alterations with a simple PCR followed by DCE. Given the procedure and the sensitivity of the *k-ras* assay, minor or moderate bleeding in the surgical area could result in a mutant fraction below the detection limit. E.g. 1 microliter of blood contains on average 5000 white blood cells, hence if 10 millilitre of blood leaked into the lavage area some 50 000 *k-ras* positive tumour cells would be required for detection. Despite this stringent limitation, *k-ras* mutant positive cells were detected in lavage fluids from the pelvic cavity. Mutated cells were found in the lavage fluid of 19 patients, which had a significant correlation to the survival. Microscopically and macroscopically surgical margins are important indicators of recurrence and survival. However, our results showed that 12 of the 19 patients positive for *k-ras* mutations were R0 stage, which was an unpredicted result. We would have expected R1 or R2 stage patients as the biggest contributors to disseminated cells in the peritoneal cavity. Cutting into the circumference of macroscopic (R2 resection) or microscopic tumour tissue (R1 resection) or

into the depth of the tumour can of course contribute to disseminated cells in the peritoneal cavity as well as by tumour infiltration of serosa or peritoneal carcinomatosis. As there were relatively more R0 stage resections in the *k-ras* negative group this should not explain our finding. However, in this case one of the R0 patients had serosa infiltration and one peritoneal carcinomatose. Lymph node metastases might contribute by a possible shedding of cancer cells when seveing an infiltrated lymphatic vessel. Therefore detection of exfoliated tumour cells may be a useful prognostic indicator based on these findings, independently of the R and N stage or other pathological features. Several other studies have performed detection of disseminated cells in the peritoneal cavity and prognostic value of these methods. Most of them have used cytological and immunohistochemistry methods. Low sensitivity and specificity might contribute to conflicting results using these methods in detection of disseminated tumour cells [163-165]. With the introduction of PCR, smaller quantities of DNA could be detected and the sensitivity and specificity be improved. The robustness of the analysis has been improved by fluorescence-based allelotyping techniques involving capillary electrophoresis [166;167].

In this pilot study we found the frequency of *k-ras* mutations in rectal cancer to be 30% and one-third of these showed *k-ras* mutations in the lavage fluid. One limiting factor of this study is that a marker is not present in about 70% of rectal cancer patients. Due to this other gene mutations need to be investigated to approximate 100% sensitivity. Pelvic lavage samples before and after tumour resection, tumour samples and blood samples from all the patients would also have contributed to increased sensitivity and a more robust study. We therefore underline that this study should merely act as a trigger for a larger multicenter study using a number of genetic markers. Due to the above-mentioned reasons sufficient number of cases allowing multiple regression analysis should allow a conclusion of importance of preoperative intraoperative contamination of tumour material.

There are conflicting results when it comes to the prognostic significance of mutated *k-ras* in the primary tumour. This is likely to arise from differences in study size, patient selection, tumour sampling, in use of archival versus fresh/frozen material, and in laboratory methods and data analyses. Mutant *k-ras* was in fact indicative of worse prognosis in CRC in some published studies [168-174], but not in others [164;175-178]. Differences among specific mutation classes were also observed independently in some studies [169;173;174;179].

Instrumentation considerations

In this thesis we have demonstrated and used an automated DCE method on a single (paper II), or a 96 capillary (paper I, III, IV, V) instrument.

We used the theoretical melting temperature corrected for chemical denaturant as a guideline for an estimated run temperature and observed differences in temperature controls in the two instruments used in this thesis. The irregularity may be explained by differences in design and size of the capillary chamber. For the single capillary instrument the ABI 310, the temperature control was satisfactory. The 310 Analyzer utilizes a ceramic heat plate to control the temperature around parts of the capillary during the electrophoresis.

For the MegaBACE 1000 irregularity in temperature was observed. This instrument has a rather large capillary chamber containing 6 arrays with 16 capillaries each. Within the chamber, the airflow can give up to 1.5°C differences in temperature between capillaries depending on the position in the chamber. To compensate for this temperature differences in the chamber, gradually decreasing temperature was used during electrophoresis in paper I. The temperature gradient makes the temperature control more robust, especially compared to CDCE where the temperature has to be strictly controlled to achieve separation [92]. This can be explained in that use of the gradient ensures that the capillary will pass through the optimal temperature for the target fragment. However, Bjørheim et al. [180] demonstrated later that neither constant temperature, nor temperature gradients allow optimal temperature distribution across all capillaries in a 96-capillary format. Consequently, temperature controls were improved by cycling the denaturing temperature around the theoretical transition state temperature. Cycling of the temperature during electrophoresis leads to almost equal conditions in all capillaries and between runs [112] (paper III, IV and V). Minarik et al. [122] have also used cycling temperature for the DCE method for mutation detection of several tumour suppressor genes. A total of 7 different somatic mutations were identified among 32 *k-ras* mutant samples, 1 inherited mutation and 5 somatic mutations were identified among 15 APC mutated samples. In paper II we used a commercial single capillary instrument. The temperature control in the ABI is not able to make a temperature gradient, and could only be adjusted with increments of 1°C. This may require more prerun optimizing before 48 samples could be analyzed without any intervention. We used POP-4TM and POP-6TM in the setup to make all parts of the protocol commercially available. Migration time between homoduplexes was prolonged with POP-6^{TM} as compared to POP-4^{TM}. This is most likely due to increased level of polyacrylamide in POP-6^{TM} [181]. DCE is

a powerful tool for analysis of DNA variation. The development of a commercial multi-CE instrument allows large-scale studies of DNA variation. However, the cost of consumables like capillary arrays and sieving matrix might limit the use of DCE in such studies. Ekstrøm and Bjørheim [182] has tested the ability of 72 different in-house formulated sieving matrices to separate PCR-amplified alleles by CTCE. They demonstrated that allelic separation is possible independently of matrix as long as the temperature, corrected for urea concentration in the polymer, is cycled in the vicinity of the Tm of the target sequence. The broad window of separation of alleles makes the method simple to apply to new fragments. This will make CTCE more accessible to the general research community (not only technology specialists), with the need only for theoretical estimation of melting temperatures as the basis for selection of cycling conditions. This is in contrast to screening methods like Single Strand Confirmation analysis (SSCP) and denaturant high performance liquid chromography (dHPLC). SSCP has no theoretical foundation to predict the separation condition, thus relying entirely upon empirical data for each fragment [183]. On the other hand, dHPLC is based upon similar statistical mechanical calculations of the transition temperature of DNA but has to accurately control the mobile phase and column temperature [184].

The costs of fluorescently labelled primers for use in PCR are another major factor in large-scale projects requiring mutation analysis in hundreds or thousands of samples.

We have therefore designed and developed a single, universal priming system for the highly specific labelling of DNA amplification products. The procedure is based on a triplex-primed PCR, employing two genome, or template, specific primers and a third labelled, universal primer. The advantage of this design is the reduction in time required to obtain labelling primer sets, the cost of primer sets, and the efficient use of labelled primers where only a limited number of genotyping applications per locus may be required.

Benesova-Minarikova,L. et al. [185] have shown a simple approach to detection of unlabeled DNA fragments through intercalation without a need for adding intercalator to the separation polymer matrix on a MegaBACE 1000.

The sensitivity is an important factor in all molecular techniques used for detection of low fraction mutations. The sensitivity of the DCE method was evaluated by mixing mutant and normal alleles in different ratios (paper I). The areas under the peaks were used as a quantitative measurement of the mutant fraction in the samples, as described earlier [90]. Loss of signal from the heteroduplex peaks was observed for mutant fractions below

4×10^{-3}. The sensitivity found is in agreement with the sensitivity reported for automated CDCE [107;120], and this sensitivity should be sufficient in many areas of mutation and SNP detection/analysis. The practical confirmation of this method, detection of *k-ras* mutations in tumours and lavage fluid was accomplished in paper V. In comparison sequence analysis of tumour DNA is a widely applied method, and is considered a direct and reliable approach. However, DNA sequencing has several disadvantages; the most notable of these is the low detection rate (25%). Samples containing large fractions of normal and fibrous tissues will interfere with accurate sequence analysis with respect to mutation detection. Direct sequencing of such samples will not reveal the mutated sequence since the detection limit is insufficient, and thus makes it difficult to identify the mutated alleles. It is therefore important to develop a method capable of immediately identifying the mutation without the need for sequencing. Bjørheim et al [111] have previously described a method for direct identification of oncogenic mutants in *K-ras* exon 1 with the DCE method.

Future Instrumentation perspectives

In this thesis the value of DCE method in terms of sensitivity, specificity, simplification and throughput has explicitly been described. However several approaches can be used to further increase the throughput capacity of this method. First, several fluorophores with different wavelength can be used in the same electrophoretic run. The capillary instruments have four channels for registration of emitted light; only two of the channels were used in the experiments reported in this thesis. Secondly this method can easily be adapted to a fully automated setup with the Caddy™, a robotic sample plate manipulator used together with the MegaBACE™ genetic analyzer [186]. And a throughput of more than 25 000 samples weekly is accomplished with a MegaBACE 4000 (384 capillaries) [187]. Furthermore development of a capillary instrument with 10 000 capillaries is under construction, and comprehensive scanning of all (25 000) genes for DNA variation will be possible in 1 000000 people [140].

Discovery of mutants with allele frequency less than 0.1% in a multicapillary instrument places great demands on sensitivity and dynamic range. With some improvements in optical detection the commercial multicapillary DNA sequencer together with fraction collection further facilitates the discovery of extremely low frequency alleles. Several groups have previously reported methods for fraction collection both in single and multicapillary

systems. However commercial multicapillary DNA analyzers are not yet equipped with automated fraction collection capabilities. This improvement of the automated capillary-based fraction collection system could lead to high-throughput mutation detection by heteroduplex analysis or constant denaturant capillary electrophoresis.

Conclusion

DCE methods were adapted for use with commercially available single-and multi-capillary instruments. The adaption of the DCE method to commercially single and multicapillary instruments has accomplished a high throughput method for detection of DNA variation. This method has shown to be cost-efficient, with high sensitivity and specificity. In this thesis we have shown that the method has several different applications, and that it is a feasible method to detect different key mutations and polymorphisms, which can affect outcome in cancer patients, as well as the pharmacogenetics of chemoprevention trials.

Further perspectives

A large number of cellular oncogenes have been identified in human tumours, including rectal cancer. To date there is no genetic marker that gives accurate information on the prognostic impact for patients with rectal cancer. New analyses of other mutations or combinations of genes that are specific for rectal carcinoma might lead to better candidates. On the basis of recent changes in genomics and high-throughput genotyping platforms, future genetic findings of association studies could have an impact on clinical care and public health screening.

The variability in treatment responses and narrow therapeutic index of anticancer drugs are some of the key challenges oncologists face. The identification of genetic variations that predict for drug response is the first step towards the translation of pharmacogenetics into clinical practice.

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