Metachronous and recurrent cancer development in colorectal neoplasia

Clinical aspects, molecular biomarkers and proteomic patterns in surveillance and risk evaluation

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Scientific environment

The research projects have been initiated and performed while working at the Departments of Pathology and Surgery, both at the Stavanger University Hospital, Stavanger, Norway.

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This thesis is dedicated to you - Annbjørg and Jonas!

Kjetil Søreide,

Stavanger, October 2007

"It is a great thing to make scientific discoveries of rare value, but it is even greater to be willing to share these discoveries and encourage other workers in the same field of scientific research"

William J. Mayo, 1861-1939
List of publications


I. INTRODUCTION

1. COLORECTAL CANCER

1.1 HISTORICAL PERSPECTIVE

Believes and mysteries surrounding cancer have evolved throughout the history. An increased understanding of the disease has resulted in improved treatment. A brief review of some of the historical milestones of (colorectal) cancer is provided:

Findings and examination of ancient mummies indicate that cancer occurred in prehistoric time. However, the first written description of cancer is found in the Edwin Smith Surgical Papyrus and the Ebers Papyrus\(^1\). These papyri are based on what was known in surgery and medicine up to 3000 BC and 1500 BC, respectively. The Edwin Smith Surgical Papyrus contains the earliest description of breast cancer, with the conclusion that there is no treatment. In the Ebers Papyrus, enlarged thyroids; polyps; and tumours of the skin, pharynx, stomach, rectum, and uterus are described. Cancer was not a prevalent disease in antiquity, as most people did not live to see old age.

People of prehistoric times believed that cancer was caused by evil spirits, natural forces, contact with wicked men, and disharmony of the planets. According to Hebrew, Greek, and Roman teachings, cancer was caused by sin, violation of religious rules, and the wrath of gods\(^1,2\).

Hippocrates (BC 460-375), a Greek physician and the “father of medicine” in the Western tradition, was opposed to superstitions and hypothesized that tumours were caused by an imbalance of the four humours: blood, phlegm, yellow bile, and black bile. Hippocrates noted that growing tumours occurred mostly in adults and the growths reminded him of a moving crab, which led to the terms carcinos (a tumour), carcinoma (a malignant tumour), and cancer (a nonhealing malignant ulcer). The hard tumour, the scirrhus, was different from carcinos and carcinoma. The black bile was particularly bad. Hippocrates wrote of dark, beef glaze-like vaginal discharge in
association with enlargement and ulceration of the uterus. He recognized cancers of
the skin, mouth, breast, and stomach. He knew about anorectal condylomas and
polyps, and recommended examination with a speculum if they were higher up in the
colon. Hippocrates summed up his recommendation for treatment by writing that
tumours that are not cured by medicine are cured by “iron” (knife), those that are not
cured by iron are cured by “fire” (cautery), and those that are not cured by fire are
incurable. For occult or deep-seated tumours, he advised not to use any treatment
because if treated, the patient would die quickly. If not treated, the patient could
survive for an extended period.

During the Roman Empire, the medical seat shifted from Greece to Rome. The
Greek physician Claudius Galen (AD 131-203) believed that accumulation of black
bile in the breast, uterus, lips, and in hemorrhoids caused cancer – a view that
mirrored the humoral model of Hippocrates. Paracelsus postulated a similar view in
the 16th century – that cancer was the cause of a fundamental mineral imbalance
within the body (the mineral theory). The accumulation of noxious substances in
blood as cause of cancer, led to the introduction of blood letting. However, Galen’s
theory was accepted as a doctrine by medical practitioners and organized religions for
sixteen centuries (2nd to 18th century), with little modification.

The 18th century saw the first theories on the local origin of cancer. John
Hunter proposed extravasated blood as a potential etiology. Use of the light
microscope in the 19th century introduced the view of a cellular origin of cancer by
investigators such as Rudolph Virchow and Karl von Rokitansky. During the 1920s,
the theory of colorectal cancer arising from neoplastic polyps developed the early
“adenoma-carcinoma-sequence”.

From the time of Hippocrates until the late 19th century, physicians and
surgeons were convinced that surgical attempts at treating colorectal cancers were
doomed to fail. This opinion stemmed from prevailing views on carcinogenesis. The
three dominant theories, the humoral, mineral, and lymph theories, held that all
cancers developed in tissue that had a diseased disposition. Thus, excision of the
gross tumour mass alone seemed unlikely to cure the patient. Consequently, surgical treatment of all cancers, and in particular colorectal cancer, was condemned.

The 19th century represented a transition period. Advances in surgical technique made excision of rectal cancers feasible. Unfortunately, classical views that resection of cancer was futile delayed the development of surgical treatment for colorectal cancer. Indeed, it was not until the late 19th century that a few individuals ignored these tenets of classical medicine and attempted local resections of rectal cancers. By the second quarter of the 20th century, a radical change occurred in the prevailing theories of carcinogenesis. Wide acceptance of the unicellular origin of cancer and the mucosal origin of colorectal cancers washed away admonitions against surgical treatment of colorectal cancers. It became axiomatic that all cancers, including colorectal cancers, could be cured surgically if treated while still a localized disease.

The rigid proctoscope was initially described in 1853, but not practiced widely before after the 1900s. The remainder of the large bowel remained inaccessible until the development of the fiber-optic endoscopes in the 1960s. Colonoscopy, as a means of evaluating the entire colon was introduced in 1969, and by 1979 the Beth Israel...
Medical Center in New York, USA had performed more than 7,000 endoscopic polypectomies. Although already introduced by Dukes in the 1920s, the polyp-carcinoma-sequence was not widely adopted before the publications by Morson in the mid 1970s.

Surgery for colorectal cancer was initially performed to relieve of intestinal obstruction, reportedly by creating stomas. Surgery for cure was made possible through the development of anti- and aseptic techniques in the latter part of the 19th century. Surgery of the intraperitoneal colon, which was more difficult to access, followed surgery of the sigmoid and rectum. Throughout the latter part of the 20th century developments in surgical technique has improved patient survival, i.e. by the “no-touch” technique reported in the early 1950s and perfected by surgeons at the Cleveland Clinic, and notably by the standards of total mesorectal excision (TME) for rectal cancers introduced by Heald in the early 1980s. The current focus is on the laparoscopic colectomy approach, allegedly also giving the patient an immunologic advance by reduced surgical stress, which is believed to positively influence the (surgical) oncological outcome.

1.2 EPIDEMIOLOGY

Colorectal cancer (CRC) is the second most frequent cancer occurring in the Western world with about 1 million new cases developed annually. In Norway, about 3,500 new patients develop CRC each year with a male:female ratio of about 1. The age-adjusted incidence has almost tripled over the past 50 years. Geographically, the risk of CRC varies (for unexplained reasons) from county to county in Norway, with the Rogaland County perceived as being a high-risk area with a high incidence of CRC. Very few cancers are diagnosed in the young (then, often associated with hereditary syndromes), and CRC is generally perceived as a disease of the old, with cumulative age-related incidence steadily increasing after the age of 50 years, with median age of those diagnosed being about 70 years. The number of cancers is
estimated to increase before reaching a plateau into the year 2020, except for an estimated continued increase in female rectal cancers.

**The most frequent incident cancers by age and sex in Norway, 2001-2005**

**MALES** all ages (61,559 cases)  
**FEMALES** all ages (56,850 cases)

Figure: Cancer incidence 2001-2005 (top) and for year 2004 (bottom) with selected, frequently occurring cancers of solid organs, including digestive organs. Developed from www.kreftregisteret.no.

**Second most frequent cancer in both genders!**
Figure: Colonic cancer. Developed from www.kreftregisteret.no.

Figure: Rectal cancer. Developed from www.kreftregisteret.no.
1.3 RISK-FACTORS

Colorectal cancer is a genetic disease, however, only 5-6% of cancers develop on a truly inherited genetic background. The most common, and best investigated, hereditary syndromes are the Familial Adenomatous Polyposis (FAP) syndrome caused by mutations in the APC gene, and the Hereditary Non-Polyposis Colorectal Cancer (HNPCC; the Lynch syndrome) caused by deficient DNA mismatch repair enzymes. Further, 10-20% of CRCs may have a “familial” clustering of cases without any detectable or known specific genetic alterations leading to this increased risk; a relative risk of 2-3 is noted in those with a first-degree relative with CRC. CRC may also develop on an inflammatory background, such as in long-standing ulcerative colitis, with cumulative probabilities for cancer development of 2% by 10 years, 8% by 20 years, and 18% at 30 years.

Nonetheless, the majority (70-80%) of CRCs are sporadic in nature, acquired through the accumulation of genetic “hits” over a lifetime. Truly, age is the single most important risk factor for developing CRC. Nonetheless, a long line of evidence indicates (as well as the geographical clustering with high incidences in the western world) that CRC is a lifestyle-related disease, with smoking, alcohol consumption, dietary factors, and physical inactivity all contributing to an increased risk.

1.4 TREATMENT

The only curative modality for CRC is surgery. New techniques give reduced surgical trauma, such as laparoscopic resection for CRC, while the indications for i.e. metastatic surgery is being widened, with improved results. Adjuvant treatment (by different regimes) is given to patients operated on for cure who have lymph node metastasis (Dukes C, stage III cancers), while palliative chemo(-radio) therapy is given to those not amenable to curative surgery, or in an attempt to down-stage the tumour for resection. In particular, the neoadjuvant radiation approach for rectal
cancer continues to be discussed. Systemic treatment for CRC has improved over the last decades\textsuperscript{26}, with a few “targeted therapies” already introduced, such as VEGF-inhibitors\textsuperscript{27-29}.

1.5 SURVIVAL

Despite an overall increase in survival over the past 50 years, largely due to improvements in surgical technique and adjuvant therapy\textsuperscript{30}, still, 40-50% of those diagnosed with CRC are expected to die within 5 years of diagnosis due to recurrent disease. Survival is stage dependant, thus emphasizing the need for prevention and early detection and treatment of disease. Many prognostic factors have been introduce and investigated over time, while truly none have persisted to overtake the TNM-system for prognostication. Some promising markers that have been investigated include microsatellite instability, thymidylate synthase expression, p53 expression and a number of other genetic abnormalities\textsuperscript{31-36}. While not perfect, the Dukes staging system guide prognostication and adjuvant treatment for most patients, with very good prognosis for Dukes A (>80-90\% 5-year survival) and lowest survival for those with distant metastasis on diagnosis (Dukes D; <10-20\% 5 year-survival).

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1.6 SURVEILLANCE AFTER SURGERY FOR COLORECTAL CANCER

Based on the notion that a large proportion of patients have recurrence despite surgery of curative intent, it has become common clinical practice to follow patients with CRC for several years following their definitive surgery and/or adjuvant therapy. Despite this widespread practice there is considerable controversy about how often patients should be seen, what tests should be performed, and whether these differing strategies have any significant impact on patient outcomes. In Norway, guidelines for surveillance after surgery in those <75 years of age have been made by the Norwegian Gastrointestinal Cancer Group (NGICG). The surveillance regimen is largely based on the diagnostic value of carcino-embryonic antigen (CEA) measured in the sera of patients.

| Surveillance, post-operative months |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 3 | 6 | 9 | 12 | 18 | 24 | 30 | 36 | 42 | 48 | 54 | 60 |
| CEA | ✦ | ✦ | ✦ | ✦ | ✦ | ✦ | ✦ | ✦ | ✦ | ✦ | ✦ |
| US of liver | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| Chest X-ray | ✗ | ✗ | ✗ | ✗ | ✗ | ✗ | ✗ | ✗ | ✗ | ✗ | ✗ |
| Colonoscopy | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ |

Figure: The Norwegian guidelines for follow-up after curative CRC surgery, developed from 38, 39.

North-American guidelines have focused on endoscopy in follow-up after curative resection for CRC 40, however, this modality received the lowest compliance in the evaluation in a defined Norwegian population 41. A recent (updated) Cochrane systematic review 42, including eight randomized studies only, suggests that there is an
overall survival benefit for intensifying the follow up of patients after curative surgery for colorectal cancer. However, because of the wide variation in the follow-up programs used in the included studies it was not possible to infer from the data the best combination and frequency of clinical visits, blood tests, endoscopic procedures and radiological investigations to maximize the outcomes for these patients. Nor was it possible to estimate the potential harms or costs of intensifying follow up for these patients in order to adopt a cost-effective approach in this area of clinical uncertainty. Thus, the surveillance after CRC resection remains an unresolved issue and a great socio-economic health burden, were best evidence suggest a positive effect of “doing something”, but just not what. Thus, it remains an unresolved issue what the optimal surveillance approach should be 42-45.
2. THE COLORECTAL POLYP

A “polyp” is defined as any lesion of the colorectal mucosa protruding into the lumen. Polyps in the colorectum are (histologically) classified as either “hyperplastic” or “adenomatous”, with various variants in between. Hyperplastic polyps in the colorectum have traditionally been regarded as non-neoplastic, but K-ras mutation is common, clonality has been demonstrated and biochemical abnormalities have been reported. Since the 1990s, this topic has received tremendous attention, and it is now clear that the “serrated adenoma” in hyperplastic polyps represents a distinct pathway (epigenetically modulated) in current colorectal carcinogenesis understanding. The polyps evaluated in this thesis contain but non-hereditary, non-inflammatory, non-serrated colorectal adenomas diagnosed in symptomatic patients with no prior cancer history.

2.1 COLORECTAL (PRE)NEOPLASIA

The traditional understanding of developing CRC is based on the concept of the adenoma-carcinoma sequence. According to this theory, benign, (pre)neoplastic adenomas of the colorectal mucosa gradually transform to invasive cancer over time. Thus, the removal of adenomas has demonstrated a preventive effect on colorectal cancer incidence in several studies. However, controversial to this issue is the notion of the so-called (and disputed) “de novo” carcinogenesis, and the more recent developed principles of aberrant crypt foci described in the colorectum.

The “de novo” carcinogenesis: According to some reports, between 20-40% of all tumours may evolve from the colorectal mucosa de novo and grow invasively into the bowel wall. Adenomatous remnants in invasive cancer have been found only in about 20% of the tumours. While the concept of de novo carcinogenesis was considered as a phenomenon related to Asian populations, it has gained more attention in the Western world during the past decade.
Aberrant Crypt Foci (ACF): The suggested first and earliest identifiable neoplastic lesions in the carcinogenetic model of the colon and rectum are so-called aberrant crypt foci (ACF). They are defined as small circumspect areas in the colorectal mucosa with enlarged crypts as compared to surrounding normal mucosa. It is thought that in the course of subsequent accumulation of biochemical and mutational changes some of the ACF develop to cancer. The progression of ACF to polyp and, subsequently, to cancer parallels the accumulation of several molecular alterations and mutations whereby a small fraction of ACF evolve to CRC. Recent data indicate that not uncommonly, some ACF bypass the polyp stage in their carcinogenesis thus reinforcing the importance of their early detection and the understanding of their pathogenesis. ACF show variable histological features, and can be grouped into differing categories by in vivo examination with high-magnification-chromoscopic-colonoscopy (HMCC). As expected, ACF are more frequently detected in distal animal and human colons coinciding with the geographic distribution of CRC. Various markers may be altered within ACF suggesting possible prospective pathological changes. These transformations may lead to the identification of the earliest pathological features initiating colon cancerogenesis. The long line of evidence developed over the past decade suggest that ACF might be an important, yet unresolved, biomarker for CRC. While endoscopic techniques continue to evolve and thus changes the ways of early detection and diagnosis of (pre)neoplasia and early cancer, the colorectal polyp continues to be the main target lesion.

2.2 CLASSIFICATION

2.2.1 Macroscopy/endoscopy

Colorectal neoplasia research has traditionally focused on exophytically growing (protruding) tumours. However, during the past decades, Japanese researchers have emphasized the importance of intramucosal premalignant and malign lesions with endophytical growth pattern and early invasion into the bowel wall. The first case of
depressed neoplastic lesions was reported by Kariya in 1977. Current concepts include both elevated, flat and depressed neoplastic lesions. As stated, high-magnification-chromoscopic-colonoscopy (HMCC) and newer techniques (such as endoscopic DBI; double band imaging) will further alter the “macro-endoscopic” picture and retrieval of (pre-)cancers in the colon.

2.2.2 Microscopy

As such, definition of neoplasia in the colorectal mucosa remains a central issue. According to the WHO classification, intramucosal neoplastic lesions are confined to the mucosal layer (i.e. mucosa, lamina propria and muscularis mucosae), and are:

- graded to the degree of *intraepithelial neoplasia (IEN)* (low-grade vs. high-grade), and

- typed by their predominant histologic *growth pattern* as tubular, tubulovillous or villous.

As depicted in this system, dysplasia is conventionally graded using a WHO two-tier system, with little/no room for “that what is in between.” Low- and high-grade IEN convey different connotations regarding cancer risk. This perspective argues that the critical differential diagnosis is the one between neoplastic and non-neoplastic epithelial cell proliferations. The stepwise, continuous model of (pre)cancer progression thus makes the relevance of IEN grading questionable. It is furthermore expected that a molecular signature will predict the propensity to invasive carcinoma more accurately than routine histopathology in the (near) future. Research in this field needs to focus on a combination of biomarkers representing the molecular spectrum of genomic and proteomic expressions.

Invasive cancer is defined as tumour invading beyond the muscularis mucosae layer into the submucosa. However, Japanese pathologists consider intramucosal lesions as early CRC based on other features than solely invasion like nuclear and
structural changes, thus possibly leading to higher incidence-figures of early malignant lesion in the colorectum. Accordingly, intramucosal lesions with high-grade dysplasia as defined by WHO may be diagnosed as intramucosal cancer according to the Japanese criteria. However, there has recently been achieved an international consensus with regard to classification of gastrointestinal epithelial neoplasia ("Vienna classification") Further, a “malignant polyp” is one containing a cancer in situ, as defined by the Haggitt’s classification. The latter lesions represent a synchronous (same time) occurrence of “cancer in adenoma”. On the other hand, a non-cancerous adenoma may predict a patient’s future risk of having new neoplasia/carcinoma develop (metachronous cancer). While several technical difficulties exist in the classification and accuracy of retrieved polyps/adenomas, ranging from the endoscopic procedures per se to the biopsy itself, it is clear that improved tools for better classification is needed.

2.2.3 Morphometry

Generally, morphometrics (from the Greek "morph," meaning shape or form, and "metron", meaning measurement) comprises methods of extracting measurements from shapes. Morphometry, as a part of quantitative pathology, has tried to classify tissue lesions in a metric (and preferably more objective) mode rather than the subjective assessment performed by pathologists. While morphometric measurements have been applied with a wide range of methods in colorectal neoplasia (from adenoma to carcinoma), there is considerably heterogeneity in the studies performed, and a general applicability and consensus for clinical practice has thus far not been reached.

2.3 PREVALENCE

The prevalence of colorectal adenomatous polyps varies widely from country to country. Among asymptomatic, average-risk patients, adenoma prevalence
averages approximately 10-20% in sigmoidoscopy studies and over 25% in colonoscopy studies, whereas the prevalence of colorectal cancer among these patients is less than 1% . These data may change in the future due to the advent of new technological approaches and, in particular, chromo- and magnifying endoscopy as well as confocal laser endoscopy. The cumulative incidence of new adenomas within 3 years after normal endoscopy averages about 7% by flexible sigmoidoscopy and 27% by colonoscopy . However, the true incidence rate is hard to estimate exactly, as it depends strongly on the definition used for diagnosis and selection criteria in the populations studied. Regional differences in adenoma prevalence rates demonstrates a clear, positive correlation with increasing age and with increasing incidence of cancer in the population under study; with 4-6% of those under 50 years having adenomas, and up to 50-60% of those >75 years having an adenoma.

2.4 DETECTION AND PREVENTION

As adenomas are believed to be the precursor to colorectal cancer, its detection and removal, or frank prevention from developing in the first place, is though of as the cornerstone in reducing the in incidence and, consequently, mortality from CRC. In this aspect, there is a plethora of approaches to achieve this; ranging from chemopreventive strategies, to occult fecal blood or molecular detection mechanisms, “virtual colonoscopy” by modern multi-slice computer tomography, and various screening programs using endoscopic techniques. While detection and removal of adenomas are feasible by endoscopic techniques, the majority of adenomas are thought to never develop into cancer. In fact, evidence suggest that a number of adenomas regress over time when left in situ. Thus, defining the “high risk” adenoma, and consequently defining the patients, requiring closer surveillance is a major research target. As such, the adenoma is a “surrogate endpoint biomarker”, however, defining characteristics within the adenoma (-patient) would further narrow the target group for enhanced surveillance.
2.5 SURVEILLANCE AFTER POLYPECTOMY

Consequently, defining high-risk adenomas is important for surveillance strategy, follow-up intervals and intervention planning according to individual risk \(^{96}\). Evidence suggests that multiplicity (≥3 adenomas), size (≥1 cm), villous features, and high-grade dysplasia are predictors of future advanced adenomas or cancers \(^{97,98}\). However, the studies from which these data derive are heterogeneous. The distinction between synchronous and metachronous CRC have not always been made, and studies have found an intriguing high rate of cancer in the early period of surveillance \(^{99,100}\), indicating a high likelihood for “missed” advanced adenomas or (pre)cancers at index colonoscopy. Yet, still, the adenoma size, numbers, and histologic grade are the features by which current surveillance is guided. While adenoma numbers can be counted and size can be measured, the histologic type and grade has to be judged — an art well, but not perfectly, performed by pathologists \(^{101}\). Few published studies stratify the incidence of advanced adenomas at surveillance colonoscopy according to index colonoscopy findings. In the future, large prospective studies or studies using pooled data from existing randomized controlled trial databases or polyp registries should be used to better define which patients are at low vs high risk for advanced adenoma recurrence. Thus, future screening and surveillance strategies should preferably be tailored after other and better surrogate endpoint biomarkers within the colorectal mucosa than the traditional adenoma features.
3. COLORECTAL CARCINOGENESIS

3.1 THE HALLMARKS OF CANCER

Cancer is now understood as the result of an accumulation of genetic alterations that allows growth of neoplastic cells with certain phenotypic hallmark characteristics\(^\text{102}\). Each tumourtype may show tissue- (and even patient)-specific molecular alterations that, however, as an endpoint goal of every neoplastic cell, serve to provide the cancer cell for:

- self-sufficiency in growth signals (i.e. through mutated oncogenes),
- insensitivity to anti-growth signals (i.e. mutated tumour suppressor genes),
- evasion of apoptosis (loss or inhibition of apoptosis signals),
- limitless replicative potential (gain of telomerase function),
- sustained angiogenesis (increased vascularity for nutrition),
- the ability to invade tissues and metastasize (“seed & soil” capacity in tissues)\(^\text{103}\).

Figure: The hallmarks of cancer.
This can be achieved through various molecular mechanisms, complicating the network and understanding of carcinogenesis. Cancer development follows a clonal evolution with continuous accumulation of mutations contributing to the acquirement of the required phenotypic cancer hallmarks. In essence, a tumour forms from any single aberrant cell, which may, through further cell division, develop into an accumulation of preneoplastic cells (i.e. polypous growth), again forming into a precursor lesion (i.e. adenoma), further acquiring malignant features (i.e. carcinoma) and then invade and spread to distant sites (metastasis, such as in liver or lungs).

3.2 COLORECTAL CANCER DEVELOPMENT

Development of CRC from an adenoma to carcinoma may take several decades. In the colorectum, a continuous turnover of the epithelium occurs, with a shift of the epithelial lining every 4-6 days. Thus, the continuous exposure to various intestinal, luminal contents, and the potential for acquiring early mutative events in cryptal stem cells, may in due time cause neoplastic transformation and cancerous overgrowth in the large bowel\textsuperscript{104}. Stem cells share many properties with malignant cells, such as the ability to self-renew and proliferate. Colorectal cancer (together with many other cancers) is believed to be a disease of stem cells\textsuperscript{105}. The gastrointestinal tract has high cancer prevalence partly because of rapid epithelial cell turnover and exposure to dietary toxins. The molecular pathways of carcinogenesis differ according to the tissue involved (e.g., pancreas, biliary tree, colorectum)\textsuperscript{17, 106, 107}, although similarities exist.

Research on hereditary cancer syndromes, including familial adenomatous polyposis (FAP), has led to advances in the understanding of the events that occur in tumour development from a gastrointestinal stem cell. The initial mutation involved in the adenoma-carcinoma sequence is in the “gatekeeper” tumour-suppressor gene adenomatous polyposis coli (APC). Somatic hits in this gene are non-random in FAP, with the type of mutation selected for by the position of the germline mutation. Clonal expansion of mutated cells occurs by niche succession. Further, expansion of
the aberrant clone then occurs by the longitudinal division of crypts into two daughter units, called *crypt fission*\(^{104, 108, 109}\). 

**Figure:** Crypt development in the intestine. From Radtke & Clevers. Self-renewal and cancer of the gut: two sides of a coin. *Science* 2005; 307: 1904-9. Reprinted with permission from AAAS.

Two theories seek to explain the early development of adenomas: the “*top down*” and “*bottom up*” hypotheses\(^{109, 110}\). Initial studies suggested that colorectal tumours were monoclonal; however, later work has suggested that up to \(\frac{3}{4}\) of early adenomas are polyclonal. Introduction of a homoygous resistance allele has reduced tumour multiplicity in the mouse and has been used to rule out random collision of polyps as the cause of these observations. It is likely that short-range interaction between adjacent initiated crypts is responsible for polyclonality\(^{105}\). Also, evolving data suggest that cancer polyclonality is caused by epigenetic disruption of stem/progenitor cells. Thus, cancer heterogeneity may be due in part to epigenetic variation in these progenitor cells, and epigenetic plasticity together with genetic lesions drives tumour progression. This crucial early role for epigenetic alterations in
cancer, is in addition to epigenetic alterations that can substitute for genetic variation later in tumour progression. Therefore, non-neoplastic but epigenetically disrupted stem/progenitor cells might be a crucial target for cancer risk assessment and chemoprevention in the future\textsuperscript{111}.

Invasion by colorectal carcinomas is characterized by an epithelial-mesenchymal transition (EMT)-like dedifferentiation of the tumour cells involving several mechanisms\textsuperscript{112}. However, a redifferentiation towards an epithelial phenotype, resembling a mesenchymal-epithelial transition, is detectable in metastases. This indicates that malignant progression is based on dynamic processes, which cannot be explained solely by irreversible genetic alterations, but must be additionally regulated by the tumour environment. In fact, there is a growing attention to the extracellular matrix which surrounds the tumour tissue, where inflammatory mechanisms and protease-systems among others are believed to foster invasiveness and metastasis in the epithelial tumour cells\textsuperscript{113-115}. At the same time, several factors in the extracellular matrix may in fact prevent cancer progression, invasion and metastasis\textsuperscript{103, 116}. Thus, the complexities herein will continue to receive much attention in the years to come.

Figure. General depiction of the extracellular matrix (ECM) and factors involved in cancer development, progression and metastasis. Developed from Søreide et al\textsuperscript{114}. 
The multigene, clonal evolution, and selection model of initiation and progression of CRC proposed by Fearon & Vogelstein originally identified the *APC* gene, genes on *18q*, and the *K-ras* and *p53* genes as those in which mutations contribute to the evolution of CRC\(^{117}\). Although confirmed by later studies, many additional genes are also involved. In fact, the recent understanding of the heterogeneity in colorectal carcinogenesis involves mechanisms related to chromosomes, to microsatellites, or to epigenetic phenomena, all distinctively driving the cells into cancer via several possible pathways of proliferation, invasion and metastasis\(^{17, 47, 48, 114, 118-124}\).

Consequently, rather than representing a linear model of required accumulative mutations in the *APC*, *K-ras*, and *p53* genes (<10% of all CRCs have all mutations) recent studies suggest they may each represent alternative, multiple mutational pathways in colorectal cancerogenesis\(^{125}\), with specific associated chromosomal aberrations\(^{126}\), and distinct clinical outcomes\(^ {123}\).

Knowledge derived from families with Familial Adenomatous Polyposis (FAP) or Hereditary Non-Polyposis Colorectal Cancer (HNPCC) helped establish the early model of colorectal carcinogenesis\(^{117, 127}\). Hereditary syndromes have germline mutations in specific genes (such as mutation in the tumour suppressor gene *APC* on chromosome *5q* in FAP; mutated DNA-mismatch repair genes in HNPCC) that greatly increase the lifetime risk for developing CRC (>80% in HNPCC) compared to the general population.

Sporadic CRC develops through randomly acquired somatic mutations in several of the same genes found in hereditary cancers\(^ {17}\). However, the rate of random mutational events alone cannot account for the number of genetic alterations found in most human cancers\(^ {128}\). For this reason, it has been suggested that destabilization of the genome may be a prerequisite early in carcinogenesis\(^ {129}\). This "mutator phenotype" is best understood in CRC, in which there are (at least) two separate destabilizing pathways – chromosomal and microsatellite instability.
3.3 GENETIC INSTABILITY & CANCER

Most cancers arise through clonal selection and waves of expansion of a somatic cell that has acquired genetic alterations in essential genes either controlling cell death or cell proliferation. Furthermore, stability of the genome in cancer cells becomes compromised because several cancer-predisposing mutations affect genes that are responsible for maintaining the integrity and number of chromosomes during cell division. Genetic instability may involve several genes at different levels; however, three major, independent or overlapping levels of instability seem to be crucial in carcinogenesis.
3.3.1 Chromosomal instability (CIN)

Chromosomal instability (CIN) is a defining characteristic of most human cancers\textsuperscript{131, 132}. Mutation of CIN genes increases the probability that whole chromosomes or large fractions of chromosomes are gained or lost during cell division. The consequence of CIN is an imbalance in the number of chromosomes per cell (aneuploidy) and an enhanced rate of loss of heterozygosity (LOH). A major question of cancer genetics is to what extent CIN, or any genetic instability, is an early event and consequently a driving force for tumour progression.
The archetypical transformation in cancer cells, including CRC, results in aneuploidy. Indeed, almost all cancer cells display a host of karyotype alterations, showing translocations, gains or losses of entire or large parts of chromosomes. Cancers do not necessarily have a higher mutation rate than normal tissue at the nucleotide level, unless they have gained a mutator phenotype through exposure to environmental stress, but rather exhibit gross chromosomal changes. Therefore, it appears that the main mechanism of tumour progression stems from chromosome instability. Chromosomal instability prevailing in cancer cells arises through several different pathways and is probably controlled by hundreds of genes. The main factors that control chromosome stability are telomere maintenance, mechanisms of cell division, and the mitotic checkpoints that govern centrosome duplication and correct chromosome segregation.

The most common genetic pathway (approximately 85% of CRCs) is characterized by allelic losses, chromosomal amplifications, and translocations. Deletion at 1p and 8p, as well as loss of heterozygosity (LOH) of 17p and 18q are frequent in CRC. Such alterations are characteristic of the chromosomal-instability pathway (CIN), also referred to as the microsatellite-stability pathway (MSS). Various techniques exist for investigating abnormalities at the chromosome level, including conventional karyotyping, fluorescence in situ hybridization (FISH), flow cytometry (ploidy analysis), comparative genomic hybridization (CGH) and spectral karyotyping (SKY), and are described more detailed elsewhere.

3.3.2 Microsatellite instability (MSI)

The second pathway (involving about 15-20% of sporadic CRCs) is referred to as the microsatellite-instability pathway (MSI). Such tumours display base-pair substitutions that are commonly found in short, tandemly repeated nucleotide sequences known (i.e. CACACACA; or [CA]₄) as “microsatellites”. This form of genetic destabilization is most commonly caused by loss of the DNA mismatch-repair function. The consequence is repeatedly inbuilt errors in microsatellite
sequences, often causing frameshift mutations and protein alterations in the microsatellite instable sequences, and thus leading to carcinogenesis.

Microsatellites are found in great number spread out over the whole DNA sequence and, due to their repetitive manner, are prone to changes during replication. The most common microsatellite in humans is a dinucleotide repeat of cytosine and adenine which occurs in several thousand locations throughout the human germ line\(^\text{153}\). Mismatches of nucleotides occur when the DNA-polymerase inserts the wrong bases in the newly synthesized DNA. Normally, when two strands of DNA replicate, nucleotide mismatches occur, but almost all such errors are quickly corrected by a molecular proofreading mechanism. The DNA mismatch repair system works as a “spell checker” that identifies and then corrects the mismatched basepairs in the DNA. However, defects in the mismatch repair mechanisms (i.e. mutated genes) lead to MSI.

Microsatellite instability was discovered to be a marker in HNPCC more than a decade ago\(^\text{153,154}\), when searching for loss of heterozygosity (LOH) in the susceptible region to find a tumour suppressor gene among dinucleotide repeats. Instead, microsatellites that had changed in length were found in all the HNPCCs – not only in the critical genetic region, but virtually everywhere in the genome of the tumour. This phenomenon was termed "replication error" (RER) and later renamed "microsatellite instability"\(^\text{153-156}\). Widespread MSI in HNPCC is associated with defective DNA-mismatch repair proteins caused by germline mutation of one of the three main genes (\textit{MLH1}, \textit{MSH2}, and \textit{MSH6}). As a consequence, the lifetime risk for developing CRC is \(>80\%\) for HNPCC offspring, compared to a lifetime risk for CRC of up to 5-6\% in the general population\(^\text{157,158}\). In addition, the risk is greatly elevated for endometrial cancer (lifetime risk of 50-60\%, compared to 2-3\% in the general population), moderately increased in ovarian and gastric cancer (12\% and 13\%, respectively)\(^\text{157,159}\), but equals the normal population for lung, prostate, or breast cancer. Deficient mismatch repair occurs in approximately 15\% of all sporadic CRC. In contrast to HNPCC, the cause in sporadic CRC is methylation of cytosine residues of the cytosine and guanine (CpG)-rich promoter sequences of \textit{MLH1}\(^\text{160-163}\). Simply
stated, the ‘epigenetic change’ affect gene function (without genetic changes) by aberrant methylation of DNA that prevents the gene (-region) from being transcribed, thus ‘silences’ the gene, and cause deficiency in protein expression (see section on “epigenetics”).

**Testing for MSI**

The choice of microsatellite markers is important for MSI testing\(^{164}\). Testing for MSI can be done at the protein level with immunohistochemistry, but genotyping by means of the polymerase-chain-reaction (PCR) is the “gold standard”. Mutations that alter microsatellite length (by deletion or insertion) are visualized as bandshifts on either electrophoresis, or by sequencing. The latter is the preferred method.

*Immunohistochemistry* (IHC), although proposed to be more cost-effective than PCR, is currently not sensitive and specific enough to be used routinely in detecting MSI in sporadic and hereditary CRC. The reasons for this are several; the heterogeneity within tumours; the weak and focal IHC staining patterns that may be associated with MSI or gene mutation, or both; variability in technical protocols for fixation and staining quality within laboratories; and differences in interpretation of results. The rate of normal IHC results (but with PCR detected mutations) ranges from 2 to 36% in the literature\(^{165-167}\), and PCR has a higher sensitivity and specificity compared to IHC\(^{168}\). One of the major problems with IHC is detection of the frequently altered *MLH1* gene mutations (sensitivity <50%).

The 1997 Bethesda Guidelines\(^{169,170}\) proposed a panel of five microsatellite markers for the uniform PCR analysis of MSI in HNPCC. This panel included two mononucleotide (BAT-25 and BAT-26) and three dinucleotide (D5S346, D2S123, and D17S250) repeats. The Bethesda guidelines have since been revised\(^{171}\), as the use of dinucleotide repeats may cause under- and overestimation of the instability-status. The revision mainly recommends the use of more mononucleotide markers in unequivocal cases (i.e. in only dinucleotide unstable cases).
Screening for HNPCC using the recommended Bethesda MSI markers are now performed in trials for detection of HNPCC\textsuperscript{172, 173}, however, the current diagnostic yield and implemented costs demand for cautious expectation at best\textsuperscript{174}. Although feasible, the choice to screen all patients who have CRC for MSI should await further evidence relating to the clinical importance and therapeutic influence of this information. Clearly, the many available techniques and numbers of markers for MSI testing make general comparison of the obtained clinical outcomes difficult in both hereditary and sporadic CRC\textsuperscript{175, 176}. However, knowledge is evolving rapidly in this field of CRC research.

**Microsatellite frequency**

For whatever the number of microsatellite markers that are used in a panel, instability in $\geq 40\%$ of markers (i.e. 2 of 5 markers in the Bethesda panel) is defined as high-frequency MSI (MSI-H), while instability in $20\%-40\%$ of markers are defined as low-frequency MSI (MSI-L). Tumours with no proven instability (or $\leq 20\%$) are termed microsatellite stable (MSS) – these tumours comprise those said to follow the CIN-pathway. The existence of MSI-L is still controversial and under debate\textsuperscript{177-181}. Complexities are related to the wide genomic distribution of dinucleotides, the yet unresolved molecular alterations that have been found in these markers, and the possible influence on carcinogenesis\textsuperscript{178, 181-183}. When many dinucleotide markers are used, a large number of CRC are graded as MSI-L\textsuperscript{181}. The select use of mononucleotide markers may avoid this problem\textsuperscript{177, 184, 185}. However, proponents of MSI-L tumours have found frequent $k$-ras mutations, as well as more frequently LOH at $5q$, $1p$ and $8p$\textsuperscript{140}, relating them to the CIN-pathway\textsuperscript{180} while this has not been confirmed in other studies\textsuperscript{181}. Interestingly, some MSI-L tumours are epigenetically silenced in the DNA-repair gene $MGMT$ (O-6-methylguanine DNA methyltransferase) more frequently than both MSI-H and MSS cancers\textsuperscript{186}, which may pose a different way to DNA repair errors. MSI-L status has been related to poor prognosis in patients with stage C cancers\textsuperscript{187}. Although techniques to more readily
detect these subtle differences are developing, such as hypermethylation of MGMT\textsuperscript{188}, the true clinicopathological yield of MSI-L status remains to be established.

**Genetic differences**

Tumours exhibiting CIN and MSI resemble each other in all but a few distinct ways. Tumours with CIN have mutations in \(p53\) and \(APC\), including gross chromosomal abnormalities. In contrast, tumours with MSI have frameshift mutations in specific target genes, such as \(\beta\)-catenin and \(TGF\beta RII\textsuperscript{189}\), and fewer mutations are found in \(k\)-\(ras\) and \(p53\textsuperscript{190}\). The same holds true for allelic imbalance at other genetic loci, such as \(18q\). Mutations of \(p53\) are associated with poor prognosis\textsuperscript{35}, explaining in one way the prognostic advantage of MSI tumours. However, MSI-positive tumours that express \(p53\) seem to have a more aggressive biology than their \(p53\)-negative counterparts\textsuperscript{191}.

Defective mismatch repair presumably facilitates malignant transformation by allowing the rapid accumulation of mutations that inactivate genes, which ordinarily have key functions in the cell. The lack of mismatch-repair proteins, fails to correct nucleotide mismatches and thus promotes mutations in other genes. However, genes carrying MSI in their own coding sequences are also involved, such as the \(BAX\) and \(TGF\beta RII\) genes\textsuperscript{192}.

A frameshift mutation inactivates the \(BAX\) gene in about 35% of all tumours with MSI. Altered \(BAX\)-expression is believed to contribute to carcinogenesis by disrupting the apoptosis pathway mediated by \(Bcl-2\)\textsuperscript{193-198}.

The \(TGF\beta RII\) gene, which encodes transforming growth factor \(\beta\) (TGF-\(\beta\)) receptor \(II\)\textsuperscript{199}, undergoes a frameshift in up to 90% of all HNPCC. This mutation leads to a disruption in the function of TGF-\(\beta\), which acts as both a tumour suppressor and promoter in CRC\textsuperscript{200-204}. TGF-\(\beta\) signalling pathway involves activation of the \(Smad\) proteins which regulate transcription. Other genes with coding microsatellites (i.e. the tumour-suppressor gene \(p16^{INK4A}\)) are mutated in mismatch-
repair–deficient CRC, but their precise roles are not well understood. More recently, the use of array technology has identified a number of genes differentially expressed in the two subtypes of CRC\(^{205-208}\).

**Clinicopathological implications**

Distinct clinical and pathological features of CRCs arising from the two separate mutational pathways have been identified\(^ {191, 209-211}\).

![Figure: Clinicopathological characteristics of MSI vs CIN tumours of the large bowel. Adopted from Søreide et al\(^ {17}\). Copyright British Journal of Surgery Society Ltd. Reproduced with permission granted by John Wiley & Sons Ltd on behalf of the BJSS Ltd.](image)

MSI is observed more frequently in women and in CRCs that occur proximal to the splenic flexure. These tumours also exhibit poor differentiation, a mucinous cell type, and frequently peritumoural lymphocytic infiltration (“Crohns-like inflammation”)\(^ {209}\).
By which mechanisms this inflammatory response may contribute to the better prognosis remains to be fully explained, but the cytotoxic effects of CD8+ lymphocytes seem to be important. Recent data suggests an interplay with TGF-β and peritumoural lymphocytes.

Furthermore, MSI tumours are usually diploid unlike the often aneuploid CIN tumours. CRC exhibiting MSI is associated with a larger size (i.e. T3 tumours) of the primary tumour, but with a more favourable stage distribution (less lymph node involvement and reduced occurrence of metastasis). The pronounced genetic instability of cells with MSI may increase susceptibility to apoptosis because of an accumulation of mutations in genes that are required for cell growth. An increased rate of mutation in other genes might lead to aberrantly expressed proteins in membranes, which may be associated with the antitumour immune response evidenced by the lymphocytic infiltrates that surround tumours with MSI.

MSI cancers have a higher incidence of synchronous and metachronous tumours. In a recent study Velayos et al explored the MSI patterns in patients with metachronous and synchronous CRC. They found that MSI occurred with equal frequency among patients with synchronous and metachronous CRCs. However, the underlying mechanism for MSI was different (loss of MLH1 expression associated with promoter hypermethylation was more common in MSI-H synchronous CRC). Observed differences in MLH1 promoter hypermethylation and patient characteristics suggested that most MSI-H synchronous CRCs were sporadic in origin.

Most importantly, patients with CRCs that exhibit MSI have longer overall and cancer-specific survival than stage-matched patients with cancers exhibiting CIN. The important contrast in survival between the two types of CRC remains unexplained. Paradoxically, colorectal cancers with MSI bear many features that are generally associated with poor prognosis, including deep tumour invasion and low histologic differentiation. However, MSI positive tumours are rarely found in hepatic metastasis from CRC.
Adjuvant chemotherapy with fluorouracil benefit patients with tumours exhibiting CIN, but apparently not those with tumours exhibiting MSI\textsuperscript{217}. However, an overall reduced benefit from adjuvant therapy in patients with MSI and CRC could not be demonstrated in a recent systematic review and metaanalysis\textsuperscript{33}, but the overall survival of MSI colorectal tumours was better. Thus, the mechanism in which MSI tends to render clinically less aggressive cancers remains an interesting but unresolved research target. Real differences in adjuvant chemotherapy response between MSI and CIN tumours remain to be demonstrated.

3.3.3 Epigenetic silencing

Aberrations in the DNA methylation patterns are recognized as a hallmark of human cancer development, and so-called “epigenetic silencing” is now recognized as a 'third pathway' in Knudson's model of tumour-suppressor gene inactivation in cancer\textsuperscript{208, 218, 219}. One of the most characteristic changes is the hypermethylation of CpG islands of tumour suppressor genes associated with their transcriptional silencing.

\textit{CpG islands} are regions where there are a large number of cytosine and guanine adjacent to each other in the backbone of the DNA (linked by phosphodiester bonds; thus named CpG). They are in and near approximately 40% of promoters of mammalian genes (about 70% in human promoters). The length of a CpG island is typically 300-3000 base pairs. These regions are characterized by CpG dinucleotide content equal to or greater than what would be statistically expected (approx. 6%), whereas the rest of the genome has much lower CpG frequency (approx. 1%), a phenomenon called CG suppression. Unlike CpG sites in the coding region of a gene, in most instances, the CpG sites in the CpG islands of promoters are unmethylated if genes are expressed. This observation led to the speculation that methylation of CpG sites in the promoter of a gene may inhibit the expression of a gene. Methylation is central to imprinting along side histone modifications. The usual formal definition of a CpG island is a region with at least 200 bp and with a GC percentage that is greater
than 50% and with an observed/expected CpG ratio that is greater than 0.6. Most of the CpG islands are associated with genes, and can be used as recognition sites for restriction enzymes.

The target genes of epigenetic silencing are distributed in all cellular pathways (e.g. apoptosis, DNA repair, cell cycle, cell adherence), and are thought of as early initiators of carcinogenesis. They are "classical" tumour suppressor genes with associated familial cancers (e.g. BRCA1, hMLH1, p16\textsuperscript{INK4a}, VHL) and putative new tumour suppressor genes which loss may contribute to the transformed phenotype (e.g. MGMT, p14ARF, GSTP1, RARB2). A tumour-type specific profile of CpG island hypermethylation exist in human cancer that allows the use of these aberrantly hypermethylated loci as biomarkers of the malignant disease. The eruption of new technologies for the careful study of the DNA methylation patterns, and their genetic partners in accomplishing gene silencing, may also provide us with new drugs for the epigenetic treatment of human tumours.

For selected genes, epigenetic changes are tightly related to neoplastic transformation in CRC. As an example, loss of the tumour suppressor gene PTEN located at 10q23 occurs through promotor hypermethylation in CRC with MSI-H. In the colon, aberrant DNA methylation arises very early, initially in normal mucosa, and may be part of the age-related field defect observed in sporadic CRC. Aberrant methylation also contributes to later stages of CRC formation and progression through a hypermethylator phenotype termed\textit{CpG Island Methylator Phenotype} (CIMP). CIMP appears to be a defining event in about half of all sporadic CRCs. CIMP-positive CRCs are distinctly characterized by pathological, clinical and molecular genetic features.

MSI in sporadic CRC usually arises because of epigenetic silencing of the DNA mismatch repair gene MLH1, and is thus associated with methylation, but the overlap of “mutator” and “methylator” phenotypes is not exact. In particular, some cancers with extensive DNA methylation do not show the mutator phenotype. Although DNA methylation is associated with a worse outcome in CRC, this adverse
prognostic influence is lost in methylated tumours with MSI\textsuperscript{228}. Collectively, these factors add a layer of complexity to the clinical, morphological, and molecular classification of CRC\textsuperscript{47}.

3.4 PROLIFERATION AND APOPTOSIS

The balance between “life and death” is essential in all tissue development, and also in development of neoplasia and cancer. Colorectal cancer has served as a model for the detection and description of several important genes and the molecular pathways they control \textsuperscript{229}. Basically these genes regulate the inner cellular network, leading to the endresult of either cell proliferation or apoptosis. However, cross-talk among several pathways exists, and the true function of certain proteins/factors may differ with “time and dose” of their presence\textsuperscript{204}. A brief outline of a few pathways are given here.
3.4.1 Proliferation

**WNT-pathway**: The “WNT”, or “wingless” (so named for the mutator effects in the fruit fly Drosophila), pathway is one important signal pathway involved in embryonic development of the intestine as well as in cancer development of the colorectum\textsuperscript{104, 230, 231}. However, several studies have shown that yet more components of colorectal carcinogenesis are linked to β-catenin pathways – thus making this protein a “linchpin” in CRC\textsuperscript{234}. The oncogenic properties of Wnt/β-catenin signaling stem from alteration in phosphorylation-dependent protein degradation and subcellular localization of beta-catenin from cell membrane to the nucleus, where it binds to T-cell factor (Tcf) to form a bipartite transcription factor. The β-catenin/Tcf complex facilitates transcription of target genes that encode effectors for activation of cell proliferation and invasion and inhibition of apoptosis, leading to colorectal cancer development\textsuperscript{235}.

*Pro-oncogenic* factors that release β-catenin from the adherens complex and/or encourage translocation to the nucleus include RAS, EGF, c-erbB-2 and others, whereas *anti-oncogenic* factors (that also inhibit nuclear β-catenin signalling) include transforming growth factor (TGF)-β, retinoic acid, and vitamin D. Association of nuclear β-catenin with the Tcf/lymphoid enhancer factor (LEF) family of transcription factors promotes the expression of several compounds that have important roles in the development and progression of CRC. This include (but are not exclusive of) such genes and proteins as c-myc, cyclin D1, cyclooxygenase (COX)-2,
matrix metalloproteinases (MMPs), and several others \cite{114, 236, 237}. Genetic aberrations of several components of the $\beta$-catenin pathways, such as Frizzled (Frz), AXIN, and TCF-4, may potentially contributes to colorectal carcinogenesis. In addition, in the tumour invasion front, stabilized and activated $\beta$-catenin interacts with other molecular pathways to facilitate tumor progression.

**KRAS-BRAF-MAPK-pathway:** K-ras is an oncogene which, when mutated, causes constitutively cell signal stimulus for cell-cycle entry and cell division. Recent evidence suggest that APC, K-ras, and p53 have different pathways and roles in CRC development \cite{125, 126, 238}. While the combination of APC/$\beta$-catenin and K-ras alterations are found in most CRCs \cite{232}, they do not necessarily show a coexistence in all tumours. However, K-ras mutation, when it occurs (in about 35% of adenomas), seems to be an early event in adenoma-carcinoma development \cite{239}, may have additional down-stream mutations in the pathways (i.e B-raf) \cite{240}, is negatively associated with microsatellite instability \cite{190}, and confer a poor prognosis in patients with CRC \cite{241}.
3.4.2 Apoptosis

Apoptosis is described by its morphological characteristics, including cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation. The realization that apoptosis is a gene-directed program (“programmed cell death”) has had profound implications for the understanding of developmental biology and tissue homeostasis, for it implies that cell numbers can be regulated by factors that influence cell survival as well as those that control proliferation and differentiation\textsuperscript{242}. Moreover, the genetic basis for apoptosis implies that cell death, like any other metabolic or developmental program, can be disrupted by mutation. In fact, defects in apoptotic pathways are now thought to contribute to a number of human diseases, ranging from neurodegenerative disorders to malignancy, and the avoidance of the “cell death programme” is regarded one of the cancer hallmarks and an important feature in CRC \textsuperscript{243, 244}. In fact, inability to escape apoptosis could possibly explain the clinical notion of “regression” in many colorectal polyps\textsuperscript{245, 246} – and, inversely, an ability to grow and progress if the apoptotic mechanisms are avoided.

To important molecules in cancer development further illustrated the importance of apoptosis control in cancer development. One was the cloning and characterization of the \textit{bcl-2} oncogene, another was the \textit{p53} tumour suppressor gene.

\textit{bcl-2} was first identified in a human leukemia line and later in follicular lymphomas. Bcl-2 promotes cell survival by blocking apoptosis. To date, at least 15-20 Bcl-2 family member proteins have been identified in mammalian cells, including proteins that promote apoptosis and those that prevent apoptosis. In addition to Bcl-2, Bcl-xL is a potent death suppressor that is upregulated in some tumour types. Conversely, Bax is a death promoter that is inactivated in certain types of colon cancer and in hematopoietic malignancies.

\textit{p53} was the first tumour suppressor gene linked to apoptosis. \textit{p53} mutations occur in the majority of human tumours and are often associated with advanced tumour stage and poor patient prognosis. By the early 1990s, \textit{p53} was established as a checkpoint protein involved in cell-cycle arrest and maintaining genomic integrity
following DNA damage. In addition, other stimuli can activate p53 to promote apoptosis, including hypoxia and mitogenic oncogenes. Moreover, several upstream and downstream components of the p53 pathway (such as Mdm-2, ARF and Bax) are mutated in human tumours.

Clearly, it is now known that mutations in many cancer-related genes in addition to bcl-2 and p53 can disrupt apoptosis during colorectal carcinogenesis. Several signal transduction pathways promote cell survival in response to growth and/or survival factors, and these pathways may be crucial in controlling cell numbers.

Molecules that regulate apoptosis can have other activities (and many of them do). For example, p53 can promote apoptosis, cell-cycle arrest and senescence such that loss of p53 function increases viability, chromosomal instability and cellular lifespan. In colon cancer, bax and p53 mutations appear mutually exclusive, consistent with a pathway relationship. In contrast, p21 which is essential for p53-mediated arrest, is rarely mutated in human tumours. Some tumour-derived p53 mutants remain capable of promoting cell-cycle arrest while losing their apoptotic potential.

A variety of signals appear important to trigger apoptosis during cancerogenesis. Extracellular triggers include growth/survival factor depletion, hypoxia, radiation and loss of cell-matrix interactions. Internal imbalances can also trigger apoptosis, including DNA damage (produced by cell-cycle checkpoint defects or exogenous toxins), telomere malfunction and inappropriate proliferative signals produced by oncogenic mutations. In some instances the apoptotic ‘trigger’ actually alleviates an anti-apoptotic signal. For example, Insulin-like growth factor (IGF)-1 promotes cell survival through the PI3K-pathway, and depletion of IGF-1 or other survival factors can trigger “death by default”. In contrast, other stimuli involve true pro-apoptotic factors; as an example, many forms of cellular stress can activate p53, which promotes apoptosis through pro-apoptotic molecules, like Bax.

The identification of apoptotic “triggers” provides insight into the forces of tumour evolution, both in initiating neoplasia development and in tumour
progression\textsuperscript{242, 244}. Other apoptotic triggers are important in tumour progression. As developing tumours outgrow their blood supply, they encounter hypoxia (low oxygen), which can activate p53 to promote apoptosis. Cells acquiring apoptosis defects (such as \textit{p53} mutations) can survive hypoxic stress, leading to a clonal expansion within the tumour. Similarly, as developing tumour cells undergo repeated divisions, telomeres are shortened until some malfunction triggers either senescence or apoptosis. Like hypoxia, p53 is required for apoptosis induced by telomere malfunction; thus \textit{p53} mutant cells survive this response and are genomically unstable. Indeed, suppression of the apoptotic response to telomere malfunction may explain why combined loss of telomerase and p53 stimulates tumour development.

This may explain why \textit{p53} mutations are usually late events in tumour development, as a cell acquiring a \textit{p53} mutation might not have a selective advantage until the developing tumour encounters hypoxic conditions or achieves sufficient telomere erosion.

Clearly, the molecular network in the regulation of apoptosis and proliferation is complex. However, a few important molecules deserve attention in the current setting.

One is \textbf{Survivin} – a 16.5 kDa protein mapped to chromosome 17q25 – which was detected only a decade ago\textsuperscript{247}. Functionally, survivin is known to inhibit apoptosis, take part in cell division and mitogenic events such as control of microtubuli connection, and enhance angiogenesis\textsuperscript{136, 248}. The expression of survivin shows distinct differences between normal and malignant tissue and, plays a causal role in CRC progression\textsuperscript{249, 250}. Recently, a possible interrelationship between survivin expression and telomerase activity has been reported\textsuperscript{249}, as confirmed in this study. Endo et al\textsuperscript{249} observed correlation between survivin and \textbf{human telomerase reverse transcriptase} (hTERT) expression in colon cancer tissues, and overexpression of survivin enhanced telomerase activity by up-regulation of hTERT expression in human CRC cells. This is in line with our findings of the co-expression of these two markers\textsuperscript{251}.
Human telomeres are composed of long repeating sequences of “TTAGGG”, associated with a variety of telomere-binding proteins. The function of hTERT as an end-protector of chromosomes prevents the chromosome from end-to-end fusion, recombination and degradation. hTERT acts as a reverse transcriptase in the elongation of telomeres, which prevent the loss of telomeres during replication.

In most tumour cells, telomeres are extremely short and stable. Telomere length is an important indicator of the telomerase activity in tumour cells and it may be used in the prognosis of malignancy, including colorectal neoplasia. hTERT activation or up-regulation causes an indefinite cell proliferation. This cellular immortalization is a potentially rate-limiting step in carcinogenesis that is important for the continuing evolution of most advanced cancers. In CRC development, hTERT activation occurs during the progression from low-grade to high-grade IEN in adenomas and increases steadily with the progression of the degree of dysplasia and invasion during colorectal carcinogenesis. Telomere instability causes increased expression of p16INK4a.
4. BIOMARKER DISCOVERY

4.1 DIAGNOSTIC ACCURACY

Diagnostic accuracy is the ability of a laboratory test to correctly classify subjects into clinically relevant groups (i.e. cancer vs. no cancer). Diagnostic accuracy refers to the quality of the information provided by the classification device (i.e. cut-off level for a continuous variable) and should be distinguished from the usefulness, or actual practical value, of the information\(^{257}\).

Diagnostic tests are usually measured and interpreted in their applicability by a number of features, including\(^{258}\):

- **Sensitivity**, or the True Positive rate, which tells how good the test is at picking up people with the condition investigated. A high sensitivity is typically preferred in a screening test to rule out people without the disease.

- **Specificity**, or the True Negative rate, which tells how good the test is at correctly defining people without the disease. A high specificity is required for diagnostic tests in order to have a low false positive rate.

- **Positive predictive value** (PPV, or the post-test probability of a positive test), which is a measure of the probability of having the condition if a person tests positive.

- **Negative predictive value** (NPV, or the post-test probability of a negative test); will address the situation “if a patient/person tests negative on a test, what is the probability of not having the condition/disease”.

- **Accuracy**, gives the proportion of all tests that have given the correct result (true positives and true negatives) as proportion of all the results.
Sensitivity and specificity are features of the test itself, and “looks backward” in that they show the probability that a person with a disease will have a positive test, rather than “looking forward” and showing the probability that the person who tests positive actually has the disease \(^{259}\).

**Likelihood ratios** (LR) is an estimate of the relative predictive value of a test (true positives/false positives), is useful in clinical practice as it indicates how likely a positive result will be found in a person with the disease compared to a person without the disease. LR of a test indicates the increase from pre-test probability (e.g. prevalence of the disease) to post-test probability. Interpretation of LRs can be used by nomograms. As a rule of thumb, LR over 10 is generally regarded as large and a conclusive change in pre- to post-test probability of having the disease. LR of 5-10 are considered moderate, and LR<2 are rarely considered important \(^{259}\). For tests with a continuous range of test-results (e.g. from 1-100), rather than a dichotomous test-result (yes/no; red/green; present/absent), the sensitivity and specificity (and the LR) heavily relies on the chosen cut-off value for dichotomization into f. ex. healthy vs diseased.

As an example, expression (positive stain) of any protein biomarker in cancerous tissues produces a continuous spectrum of test results (i.e. from 0-100%). Thus, the diagnostic properties of such biomarkers (as expressed by sensitivity, specificity, predictive values, or likelihood ratios) depend on the chosen cut-off value to differentiate between normal and disease states \(^{260}\).

**Receiver-operating characteristics curve** (ROC) analysis provides a statistical method to assess the diagnostic accuracy of a test with continuous spectrum of results \(^{261}\). The ROC curve is a graphical display of the true-positive rate (sensitivity, y-axis) and the false-positive rate (1-minus-specificity; x-axis). Each classification rule, or cut-off level, generates a point on the graph. The traditional ROC curve arises when a continuous value is measured in each subject and the classification is positive if the value is above a threshold. As the threshold varies, a new classification rule is created, and the resulting plot is a single curve. The optimal
ROC curve is the line connecting the points highest and farthest to the left-upper corner. The rationale for the optimal ROC curve is that it captures the trade-off between sensitivity and specificity over a continuous range. The area under the curve (AUC) is a measure of overall diagnostic accuracy of the test, and the cut-off value providing the highest sensitivity and specificity is calculated. Importantly, the results are independent of the prevalence of the disease. Furthermore, ROC plots occupy a central position in the process of assessing and using diagnostic tools 37, 257, 261.

4.2 SURROGATE ENDPOINTS BIOMARKER (SEPB)

Intraepithelial neoplasia (IEN): As a near obligate precursor to cancer, the intraepithelial neoplasia (IEN) is an appropriate target for intervention. Occurring in most gastrointestinal epithelial tissues as moderate to severe dysplasia, IEN shares
phenotypic and genotypic similarities with invasive disease and is on the causal pathway leading from normal tissue to cancer. In addition, IEN serves as a significant risk marker for cancer. Subjects with IEN, particularly those with severe IEN, are at significantly higher risk than unaffected individuals for developing invasive cancer in the same tissues. This risk in fact exceeds other measurable factors (e.g., age, race, and family history), with the exception of germ-line mutations that occur in genetic syndromes. IEN is also a disease in its own right, in that treatment provides clinical benefit. In standard clinical practice, surgical interventions are used to reduce the burden of IEN. This same goal of reducing IEN burden is thus also appropriate for medical (noninvasive) intervention, not only to reduce invasive cancer risk, but also to reduce surgical morbidity.

The colorectal adenoma is an IEN prototype and a good example for using IEN as surrogate end-points (SEPs). Knowledge of the biology of tumour progression therefore allows us to identify specific tests that are useful for early detection or screening. Molecular probes, for instance, could detect altered DNA shed into the faeces. Correlation of the molecular alterations with demographic data, risk factors, environmental exposure, family history, and dietary history may provide important information on the aetiology of CRC. Molecular genetic alterations could also contribute toward the assessment of risk. Risk assessment is the search for risk factors that provide the earliest evidence for the risk of cancer in persons not diagnosed with the disease. Biomarkers that are predictive of risk can potentially trigger more aggressive interventions and surveillance. Individuals who test positive for any risk marker become candidates for an intervention or for surveillance. The earliest risk factors are probably the inherited genetic defects, which is well demonstrated in the case of CRC (for example patients with HNPCC or FAP).

**Markers of risk and markers of early detection** share the same outcome, namely, the incidence of disease. However, markers of risk and markers for early detection differ in the degree of certainty they convey regarding the existence of cancer. A risk factor confers significantly less than 100% certainty of cancer within a specified time interval, whereas early detection markers confer close to 100%
certainty of cancer. Risk markers indicate that cancer is more likely to occur within a specified time in persons with the marker than in the general population. Early detection markers indicate the existence of cancer, or that cancer will occur with nearly a 100% certainty within a specified time interval.

From a screening perspective, all surrogate outcomes in individuals not diagnosed with cancer are risk factors. Colonic polyps, for instance, are a surrogate end point for screening and a risk factor for colon cancer. The elements that are necessary in order to use risk factors as surrogate outcomes in screening, early detection, or prevention interventions are somewhat different. However, for a risk factor to be a useful SEPB, it must be strongly connected to the definitive outcome, and the probability and direction of the relationship must be known. Several criteria must be met before biomarkers can serve as risk factors or as markers for early detection, for which some steps are crucial:

- the biomarker must be differentially expressed in normal, premalignant or high-risk, and tumour tissue;
- the marker and its assay must provide acceptable predictive accuracy for risk or for the presence of cancer; and
- the variance of the detection tests and the intra- and interlaboratory variance must be known.

Risk markers are usually used as surrogate outcomes to detect the effect of a prevention intervention more rapidly than waiting for the definitive outcome. These criteria can be tested and evaluated in animal models and in human tissue specimens. Today, there are very few biomarkers for risk of colon cancer, except for the colonic adenoma per se.

4.3 ENDPOINT: SYNCHRONOUS vs METACHRONOUS CANCER

Metachronous neoplasia development after having an adenoma has been investigated in a number of ways, with a large number of parameters and techniques, but unfortunately from very heterogeneous populations, and with inconsistent use of
endpoints (any adenoma, advanced adenoma, or cancer) and definition of the “metachronous interval.” “Metachronous cancer” development is (most frequently) defined as detection of a second cancer occurring beyond a 6-12 months interval after a primary, index cancer diagnosis/surgery. For cancers developed after adenoma detection and/or polypectomy a “long-term” risk or an undefined metachronous cancer risk has been stated, as opposed to “synchronous” cancers detected in the same-session endoscopic procedure. In the adenoma study, we defined metachronous cancers as cancer developing after at least 24 months interval from the index adenoma, to avoid a high incidence of missed synchronous cancers.

4.4 GENOMICS & PROTEOMICS

4.4.1 The potential

Year 2003 marked the 50th anniversary of the 1953 landmark description of the DNA double helix. The same year the Human Genome Project completed the successfully sequencing of the human genome. These are major achievements in biology and medicine in general, not the least to the understanding of carcinogenesis. New molecular insights and technologies have given clues to the initiation, early detection and possible prevention of neoplasia, prognostic and predictive markers in oncology, and targets for early detection and therapy. Modern molecular medicine is thought to overtake the current use of pathologist dysplasia classification and tumour-staging systems in the future.

The search for cancer-causing alterations within the currently known genes of the whole genome (Genomics – the study of the human genome) is complexed by the different ways the genes may be transcribed (Transcriptomics) into a variety of functionally different proteins (Proteomics – the analysis of the protein complement of the genome), which themselves over time can undergo essential functional
changes. Each has the potential for discovery of new diagnostic biomarkers, therapy targets, and predictive and prognostic features\textsuperscript{276}.

An analogue to the understanding of the genetic code is the comparison with the alphabet. As such, we have discovered the letters (\textit{the sequence}) and have thus far found a few sentences (\textit{genes that we know of}), but we have only merely begun reading the chapter contents (\textit{how genes may be transcribed}), while the books (\textit{the proteins and the metabolites}) will keep mankind reading for many centuries to come. Obviously, the human/mammalian library contains many books. In more scientific terms, the outline of the genome has enabled the study of gene products that are the focal point of proteomic studies, the effectors of the DNA\textsuperscript{277}. Thus, considering the complexities of human nature and disease processes, we have just become aware of the \textit{alphabet-code for a vast library} of knowledge.

4.4.2 The pitfalls

Genomic medicine is poised to offer a broad array of new genome-scale screening tests. However, these tests may lead to a phenomenon in which multiple abnormal genomic findings are discovered, recently coined “incidentalomics”\textsuperscript{278}, analogous to the "incidentalomas" that are often discovered in radiological studies. If practitioners pursue these unexpected genomic findings without thought, there may be disastrous consequences. One recent example of this in Norwegian medicine is the fecal gene-testing initiated for colorectal (pre-)neoplasia detection prior to any evidence that this really improve detection and work-up in symptomatic patients\textsuperscript{279}.

First, physicians, as well as researchers, will be overwhelmed by the complexity of pursuing unexpected genomic and proteomic measurements. This is underlined by the notion that according to our existing knowledge, only 1.1\% of the genome consists of exons coding for proteins, 24\% is intronic sequences and the remaining 75\% consists of intergenic DNA currently without a known function (although knowledge is evolving rapidly) in RNA-transcription or protein translation.
Second, patients will be subjected to unnecessary follow-up tests, causing additional morbidity, and potentially impaired quality of life. While molecular markers have the potential for non-invasive, large-scale screening, such as panels used for faecal detection of occult genes for colorectal cancer\textsuperscript{280, 281}, the implementation of such tools require crucial attention to every step in the implementation process, including assessment of diagnostic accuracy, reliability, validity, cost-effectiveness, and risk-benefit analysis. Commercial benefits alone should not justify nationwide implementation\textsuperscript{279}.

Third, the cost of genomic medicine will increase substantially with little benefit to patients or physicians (but with great financial benefits to the genomic testing industry\textsuperscript{279}), thus throwing the overall societal benefit of genome-based medicine into question. Several authors have discussed the basis for these concerns and suggested similar approaches through several steps that can be taken to help avoid the risks to the practice of genomic, personalized medicine\textsuperscript{148, 263, 278, 282-284}.

That molecular markers can accurately diagnose cancer have been claimed and disputed; some prominent results have not been reproduced and bias has been proposed to explain the original observations\textsuperscript{282, 285}. As new Omics-fields are explored to assess molecular markers for cancer, bias will increasingly be recognized as the most important 'threat to validity' that must be addressed in the design, conduct and interpretation of such research.

Although molecular markers will undoubtedly provide advances in diagnosis and prognosis, the degree of success claimed at present is extraordinary. In an example, the carcino-embryonic antigen (CEA) was several decades ago purported to be nearly “…100% sensitive and specific…” for colorectal cancer screening in initial research, whereas subsequent research had very different results. History might not necessarily repeat itself, but it indicates caution before making claims of success. The non-reproducibility of the CEA results was due, in large part, to the fact that individuals who were initially studied had extensive cancer, whereas individuals who were later studied had less extensive asymptomatic cancer in which CEA might not
The fact that test results vary with the ‘spectrum’ of disease might seem obvious now\textsuperscript{37, 41}, but there was little understanding in that era of the concept of spectrum of test results and of the biases that affect research about diagnostic tests\textsuperscript{282, 286}. Development of the methods and rules of evidence by which diagnostic tests are judged today occurred in part because of the CEA experience and should guide future study design\textsuperscript{264, 282, 286}.

4.4.3 Some techniques

The techniques used in modern molecular biological medicine continuous to evolve and improve, and includes a plethora of possible approaches to explore, measure and investigate the human genome and its by-products (proteins). Reviews covering several techniques have been given in more recent publications\textsuperscript{147, 148}, and but a few examples are mentioned here:

**Immunohistochemistry (IHC):** refers to the process of localizing proteins in cells of a tissue section exploiting the principle of antibodies binding specifically to antigens in biological tissues. It takes its name from the roots "immuno," in reference to antibodies used in the procedure, and "histo," meaning tissue. IHC staining is widely used in the diagnosis and treatment of cancer. Specific molecular markers are characteristic of particular cancer types. IHC is also widely used in basic research to understand the distribution and localization of biomarkers in different parts of a tissue. Visualising an antibody-antigen interaction can be accomplished in a number of ways, either by the direct or indirect method. In the most common instance, an antibody is conjugated to an enzyme, such as peroxidase, that can catalyse a colour-producing reaction.
Figure: The basic principles of immunohistochemistry.

**Tissue microarrays (TMA):** is an array-based, high-throughput technique that facilitates gene expression and copy number surveys of very large numbers of tumours. Up to 1000 cylindrical tissue biopsies (though typically lower, e.g. 40-60 per block) from individual tumours can be distributed in a single tumour tissue microarray. Sections of the microarray provide targets for parallel in situ detection of DNA, RNA and protein (IHC) targets in each specimen on the array, and consecutive sections allow the rapid analysis of hundreds of molecular markers in the same set of specimens. TMA technology is of substantial value in rapidly translating genomic and proteomics information to clinical applications. Due to variety in core diameter (i.e. from 0.6 micrometers-2.0 micrometers), number of cores (1-3 per specimen), tumour heterogeneity (invasive front, tumour centre etc) there is a risk for sampling error and low reproducibility with careless use of this technique.

Figure: Construction of tissue microarray (TMA) for high-throughput analysis of antigens.
Polymerase chain-reaction (PCR): is a biochemistry and molecular biology technique for exponentially amplifying DNA via enzymatic replication. As PCR is an in vitro technique, it can be performed without restrictions on the form of DNA, and it can be extensively modified to perform a wide array of genetic manipulations. In 1983, the PCR technique was invented by Kary B. Mullis. He received the Nobel Prize in Chemistry in 1993 for his invention. PCR is now a common technique used in medical and biological research labs for a variety of tasks (ranging from cancer/disease research and diagnosis, to genetic “fingerprinting”, paternity issues and forensic sciences). One such applicable area for the use of PCR is the testing of microsatellite instability in cancers from patients with (clinically suspected) hereditary non-polyposis colorectal cancer (HNPCC).

PCR is used to amplify specific regions of a DNA strand. This can be a single gene, just a part of a gene, or a non-coding sequence. PCR, as currently practiced, requires several basic components, including chemical components and buffers. The most important components are:

1. DNA (“template”) that contains the region of the DNA fragment to be amplified
2. One or more primers (i.e. MSI markers or the like), which are complementary to the DNA regions at the 5’ and 3’ ends of the DNA region that is to be amplified.
3. a DNA polymerase (e.g. Taq polymerase or another DNA polymerase with a temperature optimum at around 70°C), used to synthesize a DNA copy of the region to be amplified.
4. Deoxynucleotidetriphosphates, (dNTPs) from which the DNA polymerase builds the new DNA.

The PCR is carried out in small reaction tubes that are inserted into a thermal cycler that heats and cools the reaction tubes to the precise temperature required for each step of the reaction. In practice, PCR can fail for various reasons, in part due to its sensitivity to contamination causing amplification of spurious DNA products.
Because of this, a number of techniques and procedures have been developed for optimizing PCR conditions. Contamination with extraneous DNA is addressed with lab protocols and procedures that separate pre-PCR reactions from potential DNA contaminants.

Other proteomic technologies: The definition of proteomics has greatly changed over time. Originally it was coined to describe the large-scale, high-throughput separation and subsequent identification of proteins resolved by 2-dimensional polyacrilamide gel electrophoresis (2DE). Currently “proteomics” denotes to nearly any type of technology focusing upon proteins analysis, ranging from a single protein to thousand in one experiment. Proteomics thus has replaced the phrase protein science.
II. AIMS OF STUDIES

The use of colorectal adenomas and intraepithelial neoplasia (IEN) as a surrogate endpoint biomarker for colorectal cancer is extensive. It is the current target for diagnosis, therapy and prevention of colorectal cancer and its precursors. However, the diagnostic accuracy and predictive value of the IEN in colorectal adenoma for long-term, metachronous cancer development is not optimal. A major focus has been on the detection of synchronous (“same time”) cancer in patients with colorectal adenomas rather than the long-term risk of (metachronous) colorectal cancer. The majority of adenomas never progress to cancer, thus, defining patients with adenomas at high-risk for cancer development would be beneficial in surveillance after polypectomy. The objectives of the studies on colorectal adenomas were:


- to further investigate selected morphometric cell features together with a large number of cell-cycle and apoptosis-related proteins by immunohistochemistry. Again the objective was to find new and better predictors of metachronous CRC development (paper IV: Søreide K, et al. Cell Oncol 2007;29(4):301-13).

- to find optimal cut-offs for biological markers investigated in paper II and IV and validate these in a larger set of patients. The cut-offs were evaluated by receiver operating characteristics curve (ROC) analysis, and useful predictors identified by multivariate analysis. (paper V: Søreide K, et al submitted 2007).
After surgery for colorectal cancer, national Norwegian guidelines mandates systematic follow-up to detect curable, asymptomatic recurrences or curable metastatic disease. This surveillance is performed within a comprehensive program, based on serial CEA measurements and clinical visits, in addition to radiologic investigations and endoscopy. Hitherto, the effectiveness and outcome of this programme have not been evaluated in clinical practise. The objectives of the studies were:

- to evaluate the effectiveness, costs and compliance to the systematic follow-up programme recommended by the Norwegian Gastro-Intestinal Cancer Group (NGICG) in a consecutive cohort of patients undergoing surgery for CRC with curative intent (paper I: Kørner H, Søreide K et al. J Gastrointest Surg 2005;9(3):320-8).

- to evaluate the diagnostic accuracy of serial measurements of CEA to detect asymptomatic, recurrent disease amenable for secondary curative surgery. ROC analysis was used to evaluate various cut-off levels, as well as the slope of increase in CEA from post-operative baseline to the CEA value associated with diagnosis of recurrence. (paper III: Kørner H, Søreide K et al. Ann Surg Oncol 2007;14(2):417-23).

- to evaluate the role of microsatellite instability and DNA ploidy in relation to clinicopathological features, risk of any recurrence (both locoregional and distant metastasis) and disease-specific survival in a patient cohort undergoing systematic follow-up after curative surgery for CRC (paper VI: Søreide et al. submitted 2007).
III. RESULTS OF STUDIES

Studies on colorectal adenomas

In paper II (Søreide K, et al. Am J Surg Pathol 2006;30(9):1120-9) we investigated a large set of quantitative morphometric cell features within adenomas, by using digitalized objective image analysis. We evaluated the prognostic value of classical clinicopathologic features and a monotonous population of elongated cells (MPECs) in colorectal adenomas from 171 consecutively selected patients from a defined population and with long-term follow-up. Quantitative image analysis, and univariate and multivariate regression analysis were applied. Ten of 171 patients with adenomas (5.8%) developed metachronous CRC (defined as >24 mo interval and >5 cm from the index adenoma to the cancer). Median follow-up of adenomas with metachronous CRC was 68.4 and without cancer 149.7 months (range: 25 to 192 and 25 to 256, respectively). The most prognostic classical features were the localization of the marker adenoma as proximal (i.e., in the coecum through transverse colon) versus distal from the transverse colon [P=0.0003, hazard ratio (HR)=8] and the number of polyps found during colonoscopy (<or=2 vs >2, P=0.002, HR=6). Quantitative features of the MPECs included the longest nuclear axis and variance of the number of nuclei with 2 neighbors (higher and lower in cancer cases, respectively). Of the 171 adenomas, 50 (29%) had MPECs, of which 9 (18%) patients developed metachronous CRC at follow-up, contrasting 1/121 (0.8%) without MPECs (P=0.0003, HR=23). MPECs occurred in both low-grade and high-grade dysplasia, and in tubular and (tubulo) villous adenomas. MPECs had the strongest prognostic value for metachronous CRC development. Adenomas proximally located had additional value but only if they were MPEC positive (which only occurred in 5 adenomas, 3 of which (60%) developed cancer). Having more than 2 polyps also had additional prognostic value but only in MPEC-negative adenomas [10 cases; 1 (10%) developed cancer]. Dysplasia grade and histologic growth pattern had no additional
value. Thus, colorectal adenomas with subsequent metachronous cancer development can be identified more accurately with MPECs than with classical prognostic factors.

In paper IV (Søreide K, et al. Cell Oncol 2007;29(4):301-13) we investigated morphometric cell features together with a large number of cell-cycle and apoptosis-related proteins by immunohistochemistry on tissue microarrays (TMA). We assessed the differential expression of cell-cycle and apoptosis-regulating proteins and a monotonous population of elongated cells (MPECs) in colorectal adenomas. Immunohistochemistry was performed on tissue microarrays in consecutive patients having colorectal adenomas and with long-term follow-up. Influence of classic features (e.g., intraepithelial neoplasia grade, histological type, size) was examined. Of 171 patients with colorectal adenoma 86% (n=147) were eligible for study; 10 (7%) developed metachronous CRC. Median time to cancer was 69 months (range, 25–256). Median follow-up was equal for the non-cancer and cancer groups. Elevated expression of cell-cycle regulators p16\(^{INK4A}\), p21\(^{CIP1}\), and cytoplasmic/nuclear \(\beta\)-catenin correlated with increased CRC risk (all \(P<0.0001\)), as did elevated expression of the anti-apoptosis protein survivin (\(P<0.0001\)) and human telomerase reverse transcriptase (hTERT; \(P<0.001\)). Survivin, hTERT, and nuclear \(\beta\)-catenin were the most predictive molecular markers (hazard ratios [HRs]: 6.3, 9.4, and 5.8, respectively). In a combined multivariate model, MPECs had the best overall prognostic ability (HR 28.2, 95% CI: 3.6–223.0), together with survivin, and hTERT. Within adenomas containing MPECs, several molecular markers further defined high-risk patients. MPECs, survivin and hTERT may, when validated, provide information superior to conventional histology, with relevance for the clinical management of patients with colorectal adenoma.

In paper V (Søreide K, et al submitted 2007) the investigated features in paper II and IV were validated in a second and larger set of patients (n=227=), and evaluated by ROC analysis for optimal cut-offs and useful predictors identified by multivariate analysis. We sought to validate biomarkers predictive of metachronous colorectal cancer (mCRC) in patients with sporadic colorectal adenomas from 374 consecutive patients within a defined population. Risk-evaluation was performed for
patient and adenoma (i.e. grade, size, multiplicity) risk factors, morphometric nuclear axis, and immunohistochemistry (survivin, hTERT, β-catenin, p16INK4a, p21CIP1, cyclin D1). Diagnostic accuracy was assessed by receiver-operating characteristics (ROC) curve analysis, and uni- and multivariate analysis performed by Kaplan-Meier survival plot and Cox proportional hazards methods. Of the 374 patients, 26 (7%) developed mCRC with a median of 5.6 yrs (range 2-19) from index adenoma. Age ≥60 yrs, proximal location, multiplicity (≥3 adenomas), and high-grade neoplasia were independent risk factors, with high-grade IEN and proximal location the strongest on multivariate analysis (hazard ratio=HR of 4.1 and 5.2, respectively; both p<0.05). The molecular markers had significant independent value, while hTERT (HR 11.3, 95% CI 3.9-33.1; p<0.001) and survivin (HR 7.0, 95% CI 2.4-20.5; p<0.001) were the strongest, only retaining proximal location (4/16=25% with mCRC) in the combined multivariate model. The value of hTERT and survivin were retained in the validation set. The combination of survivin and hTERT yielded high mCRC risk when both were positive (15/51=29%; OR 14.3, 5.6-36.5), modest for one positive (survivin 4/90=4.4%; hTERT 4/60=6.7%) and no risk if both were negative (0/144=0%). The multivariate risk-model showed that hTERT and survivin are the best risk-predictors for long-term, metachronous CRC development in patients with sporadic colorectal adenomas.

Studies on surveillance after surgery for colorectal cancer

In paper I (Kørner H, Søreide K et al. J Gastrointest Surg 2005;9(3):320-8) the effectiveness, costs and compliance to this programme was evaluated in consecutively accrued patients undergoing surgery for CRC with curative intent. In 194 (62%) of the patients, follow-up was conducted according to the Norwegian guidelines. Twenty-one patients (11%) were operated on for curable recurrence, and 18 patients (9%) were disease free after curative surgery for recurrence at evaluation. Four metachronous tumors (2%) were found. CEA interval measurement had to be made most frequently (534 tests needed) to detect one asymptomatic curable
recurrence. Cancer-specific survival did not differ among those patients with compared without systematic surveillance after surgery. Overall compliance with the surveillance program was 66%, being lowest for colonoscopy (55%) and highest for ultrasonography of the liver (85%). The total program cost was 228,117 euro (US 280,994 dollars), translating into 20,530 euro (US 25,289 dollars) for one surviving patient after surgery for recurrence. The total diagnostic yield with regard to disease-free survival after surgery for recurrence was 9%. Compliance was moderate. The results call for a discussion on the usefulness and cost-benefit of mandating this programme at a national level.

In paper III (Kørner H, Søreide K et al. Ann Surg Oncol 2007;14(2):417-23) the diagnostic accuracy of serial measurements of CEA was performed, using ROC analysis and evaluating various cut-offs and the slope of increase in CEA. One hundred ninety-four consecutive patients surgically treated with curative intent for CRC between July 1996 and June 1999 had systematic follow-up for five years. Follow-up included radiologic imaging, coloscopy and serial CEA measurements. Complete data including CEA measurements were available from 153 patients. ROC analysis of CEA was done with regard to detection of recurrent disease. Depending on the chosen cut-off value of CEA, the diagnostic accuracy (DA) varied widely within the normal range (CEA <or=10 U/ml). CEA >4 U/ml provided the highest sensitivity (0.78) and specificity (0.91), compared to a sensitivity and specificity at the upper normal range (CEA = 10 U/ml) of 0.51 and 0.99, respectively. Thirty-three patients (24%) developed recurrence. Among 11 (5%) asymptomatic patients diagnosed by elevated CEA levels, only two patients (1.5%) were amenable to secondary curative surgery. A threefold increase of CEA in an individual patient had the same DA as the best cut-off value (>4 U/ml). In conclusion, the diagnostic accuracy of CEA in follow-up after curative surgery for CRC is influenced by the chosen cut-off value. A threefold increase of CEA may indicate recurrent disease. In conclusion, we found that the value of serial measurement of CEA was limited.
In paper VI (Søreide K et al. submitted 2007) we evaluated the effect of microsatellite instability and DNA ploidy in relation to survival and risk of recurrence or distant metastasis in patients undergoing systematic follow-up after surgery for CRC. We investigated the impact of MSI on recurrence patterns and survival in 186 patients undergoing systematic surveillance after CRC surgery with curative intent. Systematic follow-up was performed according to Norwegian Gastro-Intestinal cancer Group (NGICG) guidelines. PCR-technique was used to analyze for MSI using quasi-monomorphic markers (BAT-26, BAT-25, NR-21, NR-24, and NR-27), and image cytometry for ploidy analysis. Tumour features were investigated with regard to risk for any recurrence (locoregional or distant metastasis), time to recurrence, recurrence-free survival (RFS) and disease-specific survival (DSS) with uni- and multivariate methods. Median age at diagnosis was 67 years; median follow-up time 6.2 years. Patients with MSI (n=37; 20%) were significantly younger (median 61 yrs; P=0.016). MSI tumours were significantly more often found in proximal colon, were larger, of more invasive nature (pT3-4), were diploid and low histologic grade, and had a more advanced stage (stages II or III) than their MSS counterparts. MSI was not associated with increased risk for any recurrence (odds ratio [OR]=1.2, 95% confidence interval [c.i.] 0.6-2.4), but had a higher OR for developing locoregional recurrence (27% vs 11%, respectively; OR 2.9, 95% c.i. 1.2-7.0; P=0.016). Also, a non-significant trend towards shorter time to locoregional recurrence (P=0.060) was noted for MSI cancers. MSI-status did not influence on RFS and DSS, nor did any of the DNA histograms, including ploidy. TNM-stage with nodal status was the overall best predictor of DSS (hazard ratio [HR]=4.9, 95% c.i. 2.6-9.0; P<0.001). In conclusion, TNM-stage, explained by nodal status (pN+) had the most prognostic feature for RFS and DSS in the selected patient cohort. MSI had no prognostic value on RFS or DSS in patients undergoing systematic surveillance after curative surgery for CRC. Risk for locoregional recurrence was significantly increased in MSI, with a trend towards shorter time to locoregional recurrence. This knowledge could be of clinical importance for the choice of surveillance modality (i.e. endoscopy vs radiologic imaging).
IV. FUTURE DIRECTIONS & CHALLENGES

As depicted in the general introduction and summary of the papers in this thesis, colorectal neoplasia (both precursors and cancer) will continue to pose a great health burden in the years to come. The understanding of colorectal carcinogenesis is rapidly evolving, however, clinical translation of the basic science results are not easily overcome and may take years to implement. However, the first “targeted therapies” have evolved into clinical use based on molecular knowledge – new modes of detection and prevention are likely to follow in due course as well.

In the overall management of colorectal pre-neoplasia it appears important to be able to identify patients (with adenomas) having high-risk features for better surveillance after polypectomy. Markers that can be both risk-markers and targets for (medical preventive) therapy would be optimal for detection and prevention. In addition, if nationwide screening is implemented, risk markers beyond the traditional adenoma may be required to better address the surveillance of patients over time. Furthermore, as the population grows older, still more will live to develop colorectal cancer – post-surgery surveillance with “one size fits all” will represent a considerable socio-economic health burden if not improved by risk-stratification and tailored follow-up.

Thus, future exploration of riskmarkers in preneoplasia and colorectal cancer needs to address this based on the known and evolving genetic pathways in colorectal carcinogenesis. Together with the utilization of modern molecular techniques in genomic and proteomic sciences new markers of disease or risk will evolve.

Lastly, succeeding in this task requires collaboration between disciplines – a hurdle sometimes more difficult to overcome than understanding the human genome itself.
V. REFERENCES


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VI. ERRATA


In figure 3, the box titled “no evidence of disease” the correct number of patients should read 116 (not 186).


Table 2, the rows 5 and 6 (from the top) should read:

<table>
<thead>
<tr>
<th>No. of adenoma</th>
<th>155</th>
<th>7</th>
<th>245 (237–254)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;2</td>
<td>16</td>
<td>3</td>
<td>201 (160–241)</td>
</tr>
<tr>
<td>Size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 2 cm</td>
<td>101</td>
<td>4</td>
<td>247 (238–255)</td>
</tr>
<tr>
<td>&gt;2 cm</td>
<td>70</td>
<td>6</td>
<td>237 (222–252)</td>
</tr>
</tbody>
</table>
VII. Papers 1-6:


