Genetic and clinical prognostic markers for colorectal cancer

Marianne Aarstad Merok

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Institute of Clinical Medicine
Faculty of Medicine
University of Oslo

Department of Cancer Prevention
Institute of Cancer Research
Oslo University Hospital

Norwegian Centre of Excellence
University of Oslo

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TABLE OF CONTENTS

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Oslo, September 2013

ABBREVIATIONS

5-FU	-	5-Fluorouracil
5yOS	-	5 year overall survival
5yRFS	-	5 year relapse free survival
ACF	-	Aberrant crypt foci
AFAP	-	Attenuated Familial Adenomatous Polyposis
APC	-	Adenomatous polyposis coli (gene/protein)
BRAF	-	v-raf murine sarcoma viral oncogene homolog B (gene); serine/threonine-protein kinase B-Raf (protein)
CEA	-	Carcinoembryonic antigen
CIM	-	CpG island methylation
CIMP	-	CpG island methylator phenotype
CIN	-	Chromosomal instable/instability
COSMIC	-	Catalogue of Somatic Mutations in Cancer
CRC	-	Colorectal Cancer
CRM	-	Circumferential resection margin
CTC	-	Computed tomographic colonography
DCC		Deleted in colorectal cancer
DNA	-	Deoxyribonucleic acid
EGFR	-	Epidermal growth factor receptor
EMT	-	Epithelial-mesenchymal transition
FAP	-	Familial Adenomatous Polyposis
FFPE	-	Formalin fixed paraffin embedded
FIT	-	Fecal immunochemical test
gFOBT	-	guaiac fecal occult blood test
GTP	-	Guanosine triphosphate
HE staining	-	Hematoxylin and eosin staining
HNPCC	-	Hereditary non-polyposis colorectal cancer
IHC	-	Immunohistochemistry
ITC	-	Isolated tumor cells
KRAS	-	Kirsten rat sarcoma viral oncogene homolog (gene); GTPase KRas (protein)
LN	-	Lymph node

ABBREVIATIONS

LNR	-	Lymph node ratio
MAPK	-	Mitogen-activated protein kinases
MLH1	-	MutL homolog 1
MM	-	Micrometastases
MMR	-	DNA mismatch repair
MRI	-	Magnetic resonance imaging
MSH2	-	MutS homolog 2
MSI	-	Microsatellite instable/instability
MSI-H	-	Microsatellite instable/instability high
MSI-L	-	Microsatellite instable/instability low
MSS	-	Microsatellite stable/stability
OS	-	Overall survival
PCR	-	Polymerase chain reaction
PI3K	-	Phosphatidylinositide 3-kinases
PIK3CA	-	Gene coding for the p110-α catalytic subunit of phosphatidylinositide 3-kinase class 1A
PIP_2	-	Phosphatidylinositol-3,4,5-triphosphatate
PIP_3	-	Phosphatidylinositol-4,5-bisphosphatate
PME	-	Partial mesorectal excision
PTEN	-	Phosphatase and tensin homolog (gene/protein)
RFS	-	Relapse free survival
RNA	-	Ribonucleic acid
SSA/P	-	Sessile serrated adenoma/polyp
TGFBR2	-	Transforming growth factor beta receptor 2
TIL	-	Tumor-infiltrating lymphocytes
TME	-	Total mesorectal excision
TSA	-	Traditional serrated adenoma
TTR	-	Time to recurrence
UICC	-	Union Internationale Contre le Cancer/ Union for International Cancer Control (former International Union Against Cancer)
US	-	Ultrasonography/ultrasound

LIST OF PAPERS

I **Prognostic impact of lymph node harvest and lymph node ratio in patients with colon cancer**

Diseases of the Colon & Rectum. 55(3):307-15, 2012 Mar

Ole H. Sjo, Marianne A. Merok, Aud Svindland, Arild Nesbakken

II **Microsatellite instability has a positive prognostic impact on stage II colorectal cancer after complete resection: results from a large, consecutive Norwegian series**

Annals of Oncology. 24(5):1274-82, 2013 May

Marianne A. Merok. Terje Ahlquist. Ellen C. Royrvik, Kjersti F. Tufteland, Merete Hektoen, Ole H. Sjo, Tom Mala, Aud Svindland, Ragnhild A. Lothe, Arild Nesbakken

III **Mutations in *BRAF* and *KRAS* identify stage-specific subgroups of colon cancer patients with inferior prognosis**

Submitted manuscript

Marianne A. Merok, Stine A. Danielsen, Matthias Kolberg, Marianne Guriby, Merete Hektoen, Mette Eknæs, Aud Svindland, Arild Nesbakken, Ragnhild A. Lothe

SUMMARY

Colorectal cancer is the third most common cancer in the world with 1.2 million new cases and more than 600 000 deaths a year. In Norway the age adjusted incidence rates have more than doubled over the last fifty years, coincidental with a rise in living standard and adoption to a more modern way of life. As developing countries improve their living conditions and approach a more western lifestyle, it must be expected that they will experience some of the same development and the global burden of colorectal cancer will increase.

The best way to reduce mortality from colorectal cancer is primary prevention or early detection and surgery, but effective postoperative treatment is also a necessity. For a treatment to be useful depends on the effectiveness of the treatment itself, but also on correct selection of patients to be offered therapy.

In Norway, postoperative adjuvant treatment is offered to fit patients up to 80 years with colon cancer stage III, in addition to stage II with gut perforation or few examined lymph nodes. This implies that there are patients with colon cancer stage II who are not offered treatment, but who will experience relapse and death of their disease while at the same time a large proportion of stage III patients who is offered adjuvant treatment are cured by surgery alone and does not really need this treatment. Unfortunately, even though several pathological and molecular markers have been explored and proposed as prognostic markers, we still lack accurate methods for identifying the patients prone to relapse both in stage II and stage III. With the exception of microsatellite instability (MSI) that is now introduced as an optional marker in some guidelines, no molecular markers have yet been accepted in clinical practice.

We used a large, unselected, consecutive series of colorectal cancer patients treated at Aker University Hospital to explore the prognostic impact of the number of examined lymph nodes, lymph node ratio (LNR), MSI, and mutation status in *KRAS*, *BRAF*, *PIK3CA*, and *PTEN.* Information on lymph nodes was based on routine examination of the surgical specimens, while MSI status was determined by PCR-based fragment length analyses of the five original Bethesda markers. For the oncogenes *KRAS*, *BRAF*, and *PIK3CA*, recognized mutational hot spots were analyzed, and in the MSI tumors, three short repeats in *PTEN* were analyzed. Due to comprehensive clinical data, the prognostic impact of each marker could be calculated

controlling for other clinical and molecular variables with known or possible prognostic value.

We found that the number of examined lymph nodes increased over the study period and this contributed to improved overall survival. It is a significant prognostic marker in stage II and III, but in stage III LNR has stronger impact.

MSI has positive prognostic impact in colorectal cancer stage II. This is most relevant for proximal colon cancers since 86% of all MSI tumors are located proximal to the left flexure. The clinical relevance is limited since MSI identifies a group of patients with superior prognosis who are not offered any treatment today, but we believe these patients should be exempted from any future clinical trials of adjuvant treatment in stage II.

In the study of prognostic impact of mutations in *KRAS*, *BRAF*, *PIK3CA*, and *PTEN*, the V600E mutation in *BRAF* was identified as a negative prognostic marker in microsatellite stable (MSS) colon cancer stage II. This is clinically interesting since it identifies a group of patients who are not offered any postoperative treatment today, but has similar prognosis to stage III or worse. Since MSS tumors are fully sensitive to 5-Fluorouracil (5-FU), the basis of all adjuvant treatment regimes, it can be expected that these patients will benefit from standard treatment.

Mutations in KRAS codon 13 were identified as a negative prognostic marker in women with colon cancer stage III. Many of these patients are already included in adjuvant treatment, but due to their inferior prognosis, all should be offered a more persistent follow-up and adjuvant therapy, regardless of age.

PROLOG

A century of research on the genetics of colorectal cancer - The History of Lynch Syndrome.

"The statistical study of carcinoma is regarded by many writers as having been carried as far as it can be profitable"

A. S. Warthin, 1913

It can be argued that genetic research on colorectal cancer started in 1913. In August that year, the first documented observation of families with susceptibility for carcinoma was published in *The Archives of Internal Medicine* by A. S. Warthin, professor of Pathology [1]. He had identified 1600 cases of carcinoma in the records of the laboratory of Pathology at the University of Michigan during his time of service. Based on the recorded family history, four families with high frequency of carcinomas were identified, "Family G", "Family F", "Family P" and "Family S". The information on "Family G" was especially comprehensive due to the fact that Warthins own seamstress was a member of this family and contributed to an invaluable detailed family history. All families demonstrated a pattern of carcinoma consistent with autosomal dominant inheritance. Cancer of the mouth, lip, breast, stomach, intestines and uterus was most common and carcinomas developed at an earlier age than in the rest of the population. The idea of a hereditary basis for cancer was met with reluctance at the time. The general opinion was that cancer was caused by environmental factors alone and prevention of cancer was a major issue among surgical writers.

Aldrin Scott Warthin (1866-1931)
Professor of Pathology,
University of Michigan.

In 1925 Warthin published an updated family tree of "Family G" [2]. The ancestor in this family was a German settler who died of abdominal cancer at

the age of 50. By 1925 he had 144 descendants and among the 88 who had reached adulthood, there were 28 cases of carcinoma (32%). Fifteen relatives had cancer in the intestine or stomach, while twelve had cancer in the uterus and one in the ovary. Warthin concluded that there was a strong suggestion of familial susceptibility of cancer. Another update of "Family G" was published in 1936 by Hauser and Weller with the same conclusion [3].

In 1966, H. T. Lynch described "the cancer family syndrome" in two families [4]. The syndrome was characterized by increased occurrence of adenocarcinomas, primarily in colon and endometrium, increased incidence of multiple primary neoplasms, and young age at onset, following a pattern of autosomal dominant inheritance. After these publications, Lynch was asked to audit the information on "Family G" and in 1971 he published the third update, almost sixty years after Warthins original paper [5]. The kindred now counted more than 650 descendants of which 95 had been diagnosed with cancer. By 1971, several other familial cancer syndromes had been clinically described like Retinoblastoma, the Familial Adenomatous Polyposis (FAP), and Neurofibromatosis. However, the mechanism of inheritance was unknown and there was still some resistance towards the idea of a genetic basis of cancer.

During the 70's and 80's, the knowledge in this field boomed. In 1971 Knudson published his statistical study on mutation rates in retinoblastomas [6]. The gene responsible for this malignant eye disease in children was connected to chromosome 13q14 by Yunis et al. in 1978 [7] and was cloned by Friend at al. in 1986 [8]. The gene responsible for FAP was linked to chromosome 5q21 by Herrera at al. in 1986 and was cloned in 1991 by Kinzler et al. [9, 10]. The disorder Neurofibromatosis was described as a clinical syndrome by the German pathologist Friedrich Daniel von Recklinghausen as early as 1882 and an autosomal dominant inheritance was demonstrated in the 1950's. The genetic changes responsible for the syndrome were mapped to the long arm of chromosome 17 by two different groups in 1987 [11, 12]. These findings were later confirmed by an international collaboration which identified 17q11.2 as the affected locus [13] and the gene was cloned in 1990 [14].

These and other advances regarding the genetic basis of disease, contributed to an acceptance of the theory of a hereditary form of non-polyposis colorectal cancer and increasing interest in the field. In 1984, Boland and Troncale introduced the term "Lynch syndrome" [15]. "Lynch syndrome I" included patients with a familial susceptibility for colorectal cancer at young age without a history of polyposis. Inheritance followed an

autosomal dominant pattern and was associated with multiple, primary neoplasms, usually in the proximal colon. "Lynch syndrome II" was analog to "cancer family syndrome" and included patients with endometrial or ovary carcinoma in addition to colorectal neoplasms. Lynch on the other hand, introduced the term "Hereditary Non-polyposis Colorectal Cancer" (HNPCC) in 1985 and the terms have been used as synonyms since [16].

Albano et al explored the natural history of hereditary colon cancer in 1982. They found younger age at onset, more tumors located in the proximal colon and more synchronous tumors in the hereditary patients compared to population-based data from the American College of Surgeons (ACS) [17]. There was significantly better prognosis in the hereditary group with 5 year overall survival of 52% as opposed to 35% in the ACS-data. In 1986 Mecklin and co workers gave the first detailed description of the histopathology in colorectal tumors from patients with "the cancer family syndrome". They confirmed the proximal location and found significantly higher proportion of poorly differentiated or mucinous carcinoma in hereditary tumors than in the control group [18].

To promote further research in this field, cooperation between different research groups were needed and "The International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer" (ICG-HNPCC) was founded in 1990. Their first meeting in Amsterdam in 1990 resulted in the Amsterdam criteria, the first set of clinical criteria defining the HNPCC-syndrome [19]. These should form the foundation for research on the genetic basis of HNPCC and were known as the "3-2-1 rule" (Table 1).

Table 1

Amsterdam criteria (1990)
At least **3** relatives with histologically confirmed colorectal cancer, 1 of whom is a first degree relative of the other 2; familial adenomatous polyposis should be excluded.
At least **2** successive generations involved.
At least **1** of the cancers diagnosed before age 50.
All criteria must be fulfilled to be included in further analyses.

In 1993, the three initial articles describing microsatellite instability in a subgroup of colorectal cancer, were published by Thibodeau, Aaltonen and Ionov respectively [20-22]. Thibodeau also found a significant association to good prognosis, a finding also demonstrated by Lothe et al the same year [23]. The latter study was done in a population-based series of

true sporadic cases. The same research team also demonstrated MSI in sporadic gastric and endometrial carcinomas, both cancer types characteristic of the HNPCC syndrome [24].

Within months, the MSH2 gene had been cloned and identified as the gene on chromosome 2p responsible for HNPCC [25, 26], and soon after mutations in the MLH1 gene were also linked to HNPCC. It was concluded that HNPCC could be caused by any mutation in the mismatch repair genes (MMR) leading to defect mismatch repair and microsatellite instability [27-29]. It has later been shown that MSI in sporadic colorectal cancer is caused by alterations in the same genes, in particular silencing due to methylation of the promoter of *MLH1* [30], but somatic mutations of the MMR genes are also described [31].

After the identification of the genetic basis of HNPCC, the mutations were identified in affected individuals and it was established that extracolonic cancers and synchronous/ metachronous tumors were a part of the syndrome. The Amsterdam criteria did not include these events and demonstrated also poor sensitivity in small kindreds. To better identify patients who should be tested for these germ line mutations, an alternative set of clinical criteria was developed in 1996 at an international workshop on HNPCC hosted by the National Cancer Institute. The Bethesda guidelines included the Amsterdam criteria, but added several other criteria to identify patients with extracolonic cancers and alternative presentations of HNPCC [32] (Table 2).

Table 2

The Bethesda Guidelines for testing tumors for microsatellite instability (1996)
1. Individuals with cancer in families that meet the Amsterdam Criteria.
2. Individuals with two HNPCC-related cancers, including synchronous and metachronous colorectal cancers or associated extracolonic cancers (endometrial, ovarian, gastric, hepatobiliary, small bowel, or transitional cell carcinoma of the renal pelvis or ureter).
3. Individuals with colorectal cancer and a first-degree relative with colorectal cancer and/or HNPCC-related extracolonic cancer and/or a colorectal adenoma; one of the cancers diagnosed at age <45 y, and the adenoma diagnosed at age <40 y.
4. Individuals with colorectal cancer or endometrial cancer diagnosed at age <45 y.
5. Individuals with right-sided colorectal cancer with an undifferentiated pattern on histopathology diagnosed at age <45 y.
6. Individuals with signet-ring-cell-type (> 50%) in colorectal cancer diagnosed at age <45.
7. Individuals with adenomas diagnosed at age <40 y.
A patient fulfilling any of the criteria above should be included in further analyses.

The guideline also suggested that a minimum of four markers should be analyzed and that instability should be defined as alteration in at least two markers. The guidelines did not however include any recommendations for which markers to include in the panel and the first set of defined international criteria for determination of microsatellite instability was not published until 1998 [33]. The reference panel of microsatellite markers included two mononucleotides (BAT 25 and BAT 26) and three dinucleotides (D2S123, D5S346 and D17S250). Instability in two or more markers should be regarded as microsatellite instability high (MSI-H), instability in one marker as microsatellite instability low (MSI-L) and tumors with stability in all five markers as microsatellite stable (MSS).

In 1998 the Amsterdam criteria were revised to meet the criticism of the criteria being too strict [34] (Table 3). And in 2002 the Bethesda guidelines were revised as well [35] (Table 4). The revised Bethesda guidelines introduced immunohistochemical (IHC) analyses of MLH1 and MSH2 as a more accessible alternative to mutation analyses in the evaluation of MSI in tumors from patients fulfilling the clinical criteria for HNPCC. The revised guidelines have been reported to have higher sensitivity in detecting individuals and families at risk of HNPCC than the Amsterdam II criteria, but are more complex [36].

The clinical criteria use family history to identify patients and families at risk. Because of major challenges in documenting a reliable family history, especially in smaller families, this can be a problem. Other methods for identification of MSI-tumors have therefore been proposed. The MsPath (MSI by pathology) model combines age, tumor grade, Crohn-like reaction and tumor infiltrating lymphocytes (TIL) and is reported to have a sensitivity of 93% and a specificity of 55% [37]. However, advanced tumor grade with poor differentiation is more prominent in sporadic cases of MSI tumors than in HNPCC, resulting in a lower sensitivity for hereditary MSI cancer than sporadic cancer for this method. If the goal is to identify all patients with HNPCC, it has been recommended that all patients with colorectal cancer should be tested for MSI and, if positive, for germ-line mutations of the MMR genes [38]. A consensus on a recommended procedure for this purpose has not yet been reached to our knowledge.

As our comprehension of HNPCC has increased, the term itself has proven to be misleading and confusing. It is now argued that the syndrome of hereditary cancer characterized by germ-line mutation in one of the MMR genes resulting in MSI-tumors should be termed “Lynch syndrome”. Patients and families that fulfill the Amsterdam criteria I but with

microsatellite stable tumors, should be referred to as "Familial colorectal cancer type X" [39, 40].

What about “Family G” and Warthins seamstress who participated in the initiation of this research? The seamstress unfortunately died of adenocarcinoma of the uterus at the age of 35, just before Warthin published the first update on the family in 1925. Seventy five years later, in 2000, a member of “Family G” was finally proven to be a carrier of a germ-line mutation in MSH2, the gene most commonly mutated in Lynch syndrome [41].

Table 3

Amsterdam criteria II (1998)
3 or more relatives with an associated cancer (colorectal cancer, or cancer of the endometrium, small intestine, ureter or renal pelvis). 1 should be a first-degree relative of the other two. Tumors should be verified by pathologic examination. Familial adenomatous polyposis (FAP) should be excluded in cases of colorectal carcinoma.
2 or more successive generations affected.
1 or more relatives diagnosed before the age of 50 years.
All criteria must be fulfilled to be included in further analyses.

Table 4

The Revised Bethesda Guidelines (2002)
1. Colorectal carcinoma (CRC) diagnosed in a patient who is less than 50 years old.
2. Presence of synchronous or metachronous CRC or other Lynch syndrome-associated tumors, regardless of age.
3. CRC with high microsatellite instability histology diagnosed in a patient less than 60 years old.
4. CRC diagnosed in one or more first-degree relatives with a Lynch syndrome-associated tumor, with one of the cancers being diagnosed at less than 50 years of age.
5. CRC diagnosed in two or more first-degree or second-degree relatives with Lynch syndrome-associated tumors, regardless of age.
A patient fulfilling any of the criteria above should be included in further analyses.

INTRODUCTION

Cancer

Clonal development

Cancer is a result of an accumulation of genetic and epigenetic changes in a clone of cells, disrupting the regulation of basic cellular features [42, 43]. A theory of the genetic nature of cancer was proposed by the German biologist Theodore Boveri in 1914 in his article "Concerning the Origin of Malignant Tumours" [44]. He argued that cancer starts with a single cell that acquires the ability of uncontrolled division. The idea of cancer as a monoclonal disease gained strong support in the years to come, but the underlying genetic instability resulting in an accumulation of genetic alterations in the progeny was for a long time poorly understood. In 1976, Nowell suggested that cancer development follows the rules of evolution, implying that any change causing a survival benefit will be selected for, resulting in clonal expansion [45, 46]. This also means that a primary tumor to some extent will be genetically heterogenic.

Molecular development

Genes involved in cancer development can broadly be classified as tumor suppressors or oncogenes. The tumor suppressors include genes that contribute to normal homeostasis in the cell and typically constrain cellular proliferation [47, 48]. Inactivation can therefore lead to malignant transformation [49]. The proto-oncogenes stimulate proliferation and promote

Epigenetics has been defined amongst others by Andrew Feinberg:

"Cellular information, other than the sequence itself, that is heritable during cell division" [50].

There are several types of epigenetic changes observed in cancer; global hypomethylation, hypermethylation of specific CpG sites, loss of imprinting, and histone modifications. These changes modify expression of genes, and by silencing of tumor suppressors or activation of oncogenes they can contribute to carcinogenesis. Both genetic and epigenetic changes in cancer target the cellular capabilities described as Hallmarks of cancer.

malignant development when activated into oncogenes by various mechanisms [51, 52] and are appealing targets for cancer treatment [53]. Both genetic and epigenetic changes (see box) can alter the expression and normal function of tumor suppressors and proto-oncogenes.

Hallmarks of cancer

In 2000 Douglas Hanahan and Robert A Weinberg described six central cellular capabilities that are necessary for cancer development, and called them "The Hallmarks of cancer" [54], including sustaining proliferative signaling, inducing angiogenesis, evading growth suppressors, resisting cell death, enabling replicative immortality, and activating invasion and metastasis.

In 2011, the list of Hallmarks were revised and two enabling characteristics were added; genome instability and mutation, and tumor-promoting inflamation. Two emerging hallmarks were also included; deregulation of cellular energetics and avoidance of immune

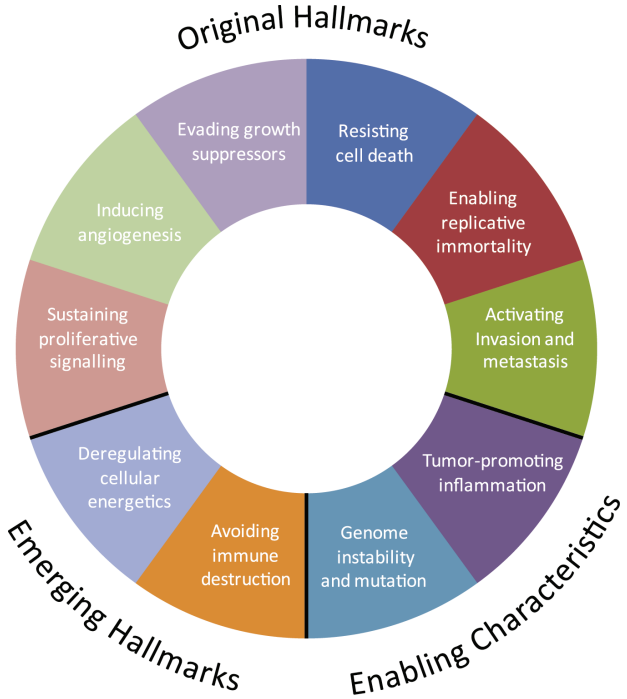

Figure 1. Hallmarks of cancer. Modified after Hanahan and Weinberg [54]

destruction (Figure 1) [55]. Equally important, the role of the tumor microenvironment was discussed, as the extracellular stroma and neighbouring cells both can contribute to carcinogeneseis or suppress cancer development. The observed changes in the microenviroment and neighbouring cells can be caused by the cancer cells themselves to promote further growth of the tumor or be the result of the organisms defence mechanisms against cancer. This implies that the different cancer promoting capabilities should not be regarded a result of changes only in the cancer cells, but a result of the sum of changes in the cancer cells and the microenviroment. This places the "Hallmarks of cancer" in the tumor as a whole, and not solely in the cancer cell as earlier described/assumed.

Colorectal cancer

Epidemiology

Almost 13 million people were diagnosed with cancer worldwide in 2008 and 7.6 million died, the majority living in developing countries [56]. Colorectal cancer is the third most common cancer in males and the second in females with a total of 1.2 million new cases and more than 600 000 deaths a year. The incidence is expected to raise as developing countries approach a more western life style [57].

In 2010, 3872 persons were diagnosed with colorectal cancer in Norway, almost five times the average number for 1956-1960 of 791 persons a year. This is partly due to an increasing population and rise in life expectancy, but the age adjusted incidence of colorectal cancer has also more than doubled over the last 50 years from 16.4 to 43.2 per 100 000 for males and from 14.4 to 35.1 per 100 000 for females [58]. The incidence is now among the highest in the world and colorectal cancer is the second most common cancer in both genders after breast and prostate cancer [59] The incidence is somewhat higher in males with a cumulative risk of colorectal cancer by 75 years of 5.1% compared to 4.1% for females. However, after a steady increase in age adjusted incidence over many years, the rates have been more stable for the last decade (Figure 2) [58].

Of the 11 036 persons who died of cancer in Norway in 2008, 1 589 died of colorectal disease. The mortality was slightly increasing up to 1990, but has since been stable or even declining, especially in rectal cancer. A likely cause of the positive development in rectal cancer is the introduction of total mesorectal excision, neoadjuvant treatment and increased specialization [58].

The 5-year relative survival has increased steadily over the last 50 years (Figure 2). Prognosis is closely associated to disease stage and 5-year relative survival is 80-90% for localized disease (stage I-II), 70-80% for regional disease (stage III) and 10-15% for metastatic colorectal cancer (stage IV) [58]. The observed improvement in survival could therefore be caused by earlier detection. However, a stage migration from local to regional disease is observed for the last fifty years. In 1956-60 localized, regional and metastatic disease accounted for 45%, 28% and 27% respectively compared to 22%, 55% and 22% in 2006-10. This migration might be explained by an increasing focus on resection and examination of mesocolic and mesorectal lymph nodes, resulting in more radical surgery and

a more thorough histopathological examination of the resected tissue and therefore more accurate staging. The improved surgery, introduction of adjuvant treatment, more aggressive treatment of metastatic disease and improved supportive care have all contributed to the increased relative survival observed for the period.

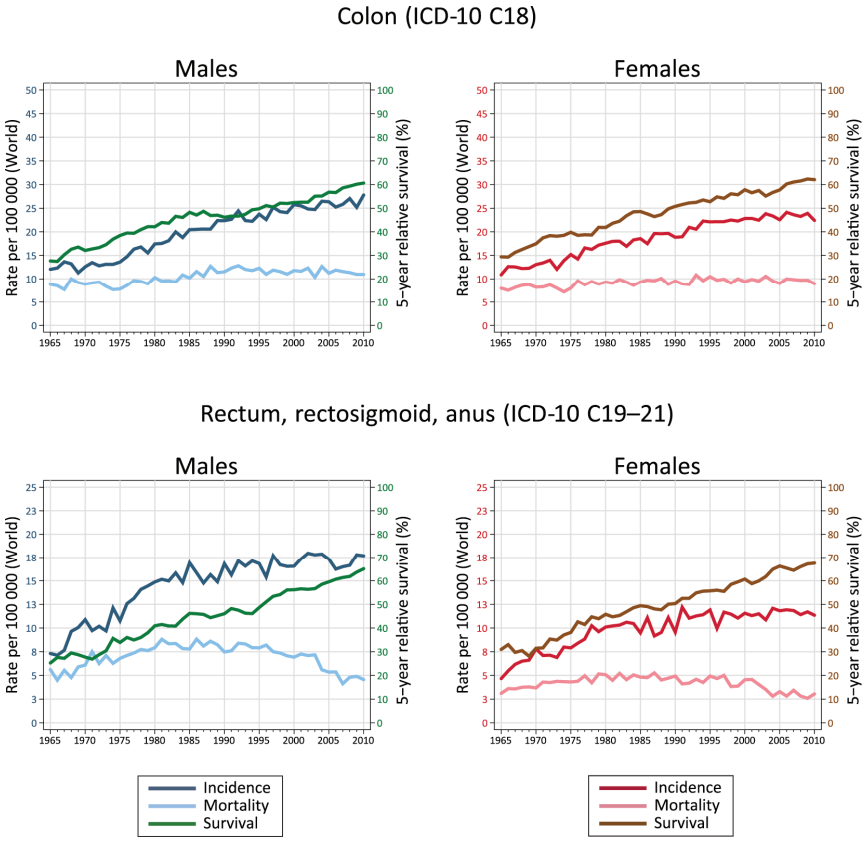

Figure 2. Trends in incidence and mortality rates and 5-year relative survival proportions. From ref [58]; «Cancer in Norway 2010», Cancer Registry of Norway.

Hereditary colorectal cancers

The majority of colorectal cancers is sporadic and arises in patients without a family history of the disease. However, up to 30% of the tumors arise in patients who report of frequent or early-onset disease in close family members. These patients are assumed to have a genetic disposition, but the genetic alteration responsible, is identified in only 2-5% of colorectal cancers [60]. Table 5 gives an overview of the recognized hereditary colorectal cancer syndromes with an identified gene defect [61-63].

Screening/Diagnosis

The most reliable route to full recovery from colorectal cancer goes through resection of all tumor tissue. Primary prevention of the cancer or detection at a stage where complete resection is possible must therefore be achieved to reduce mortality. The development from normal colon mucosa through endoscopically detectable polyps to invasive cancer takes a minimum of 5 years [64]. This gives a window of opportunity for screening, but there is debate over which modalities that are most cost-effective in this setting.

Tests for occult blood. The standard guaiac fecal occult blood test (gFOBT) detects heme, but is not specific for human blood. Food modifications are therefore necessary prior to testing. Sensitivity for colorectal cancer and advanced polyps in a set of three tests varies from 35% to 80% and 30% to 70%, respectively [65] and annual or biannual screening reduced the mortality of colorectal cancer with 15% to 33% in three large randomized controlled trials [66]. The fecal immunochemical tests (FIT) detects human globin and does not require diet or drug restrictions. It provides higher sensitivity and specificity than gFOBT and should be the preferred test in a screening setting [67].

Fecal DNA. Stool contains exfoliated cells from the gut, including cells from neoplastic lesions. DNA from these cells can be extracted and in 1993, Sidransky et al were successful in detecting mutated KRAS oncogene in stool samples from 8/9 patients with KRAS positive lesions [68]. Four other frequently altered genetic markers in colorectal cancer (APC, p53, BAT-26, Long DNA) were later included with KRAS in a test panel of totally 21 mutations. In spite of optimistic results in smaller series [69], the panel showed a sensitivity of only 52% and 18% for cancer and advance adenoma respectively in a population of asymptomatic persons at an average risk of colorectal cancer. The specificity was 94% [70]. Later modifications in sampling and DNA analyses led to a sensitivity of 73%, but in lack of validation in a screening cohort, the overall test performance is uncertain [67, 71].

Table 5. Hereditary colorectal cancer syndromes with affected genes and clinical features.

Syndrome	Affected gene	Clinical features
Lynch syndrome (HNPCC)	MLH1, MSH2, PMS2 or MSH6 (EPCAM)	No polyposis. 80% lifetime risk of colorectal cancer, mean age 44 years. Similar risk for cancer in the endometrium and ovaries. Increased risk of cancer in the stomach, urinary tract, small bowel and central nervous system.
FAP (Classic Familial Adenomatous Polyposis)	APC	Develop adenomatous polyps (> 100) in colon and rectum after adolescence. Carcinoma diagnosis by mean age of 35-40 years. Also polyps/carcinoma in the duodenum, the stomach and hypertrophy of retinal pigment epithelium.
Gardner's syndrome	APC	Phenotypic variant of FAP: Same features in addition to epidermoid cysts, desmoid tumors and osteomas.
AFAP (Attenuated Familial Adenomatous Polyposis)	APC	Less pronounced phenotype of FAP with as few as 10-20 adenomatous polyps by 50 years of age. Carcinoma by mean age of 55 years.
Turcot's syndrome	APC, MLH1 or MSH2	Multiple adenomatous colon polyps and increased risk of colorectal cancer. Increased risk of medulloblastomas (associated with APC mutation) and glioblastoma multiforme (associated with MMR mutations)
MAP/ FAP2 (MUTYH-associated polyposis)	MUTYH	Variant of AFAP. Autosomal recessive inheritance pattern. Usually no family history. Few colorectal polyps. Colorectal cancer after 45 years of age.
Peutz-Jeghers syndrome	LKB1/STK11	Hamartomatous polyps in the GI tract and mucocutaneus lesions of hyperpigmentation in the mouth and on the hands and feet. Increased risk of carcinomas in the pancreas, liver, lungs, breast, ovaries, uterus and testis. Cumulative risk by age of 70 for all cancers is 85%.
Cowden disease	PTEN	Small benign hamartomas in skin and mucous membranes as the mouth and GI tract. Increased risk of cancer in breast, thyroid, uterus and kidney. Cumulative risk of cancer by age of 70 is 89%.
Juvenile polyposis syndrome	SMAD4 or BMPR1A	Multiple polyps, sessile or pedunculated hamartomatous, primarily in colon and stomach. 60% risk of colorectal cancer by 60 years. Increased risk of cancer in stomach, small bowels and pancreas.

Tumor DNA extracted from feces can also be analyzed in terms of methylation, utilizing the observed hypermethylation at specific CpG Islands in the detection of neoplasms [72]. The vimentin gene demonstrated aberrant methylation in colorectal cancer with a sensitivity and specificity of 77% and 83% respectively [73] and a single-gene test, ColoSure, is commercially available [74]. It has however not been approved by the Food and Drug Administration (FDA) due to missing documentation of effect. Multi-gene-panels of methylated CpG Islands are believed to make more robust tests and their diagnostic value have been demonstrated in tissue [75]. These panels are not yet commercially available, but are undergoing clinical trials.

Endoscopy. Colonoscopy is regarded as the gold standard in diagnosis of neoplastic lesions in the large gut and is the final assessment step in all screening programs. There are no prospective, randomized controlled trials of colonoscopy in a screening setting yet, but a multinational initiative organized from Norway will hopefully change this [76]. For a successful colonoscopy, the sensitivity for colorectal cancer is close to 100%. For adenomas ≥10mm a miss rate of 6% has been reported while the miss rates for polyps 6-9mm and ≤5mm were 13% and 27% [77]. Colonoscopy enables both detection and removal of neoplastic lesions and is associated with a decrease in both incidence and mortality of colorectal cancer in case control studies [78-80].

However, a colonoscopy implies extensive bowel preparation the day before examination and many patients find this uncomfortable as well as the examination itself. A complete colonoscopy with adequate inspection of all sections is not always possible due to ineffective bowel preparation or challenging anatomy, resulting in repeated or alternative examination. Even when inspection of the proximal colon is possible, there is a risk of missing sessile adenomas. The risk of perforation during colonoscopy in a screening setting is low, approximately 0.1% [67]. It takes extensive training to be able to perform the procedure safe and effectively, and shortage in trained examiners can be an issue.

Flexible sigmoidoscopy includes examination of the rectum and distal colon to the splenic flexure. If any polyps are detected, a full colonoscopy is indicated, but the proximal colon is not routinely inspected. Screening sigmoidoscopy is therefore usually combined with an annually test for occult blood in the feces. A case-control study from 1992 demonstrated a 60% reduction in mortality from cancer in the distal colon and rectum for 10 years after sigmoidoscopy alone [81]. In a Norwegian randomized controlled screening trial, mortality for colorectal and rectosigmoid cancer were reduced by 59% and 76% respectively, but only

in those who attended. For the whole "intention to screen" group, only a trend towards reduced mortality were observed [82]. The bowel preparation prior to sigmoidoscopy is less extensive than for colonoscopy with a same-day enema. The examination is also easier to perform, takes less time and is less uncomfortable.

Computed tomographic colonography/ Virtual colonoscopy. Alternative modalities in colorectal cancer screening are Computed Tomographic Colonography (CTC) and virtual colonoscopy. Virtual colonoscopy use data from an advanced CTC to make 2D and 3D pictures which put together make a virtual colonoscopy that can be interpreted to identify neoplastic lesions. Results from case control studies have been diverging with reported sensitivity for lesions ≥10mm from 53% (CTC) to 94% (virtual colonoscopy). Specificity is more stable around 95% [83, 84]. A meta-analysis from 2005 concluded that sensitivity of CTC for lesions ≥10mm and ≥6mm was 93% and 86% respectively with specificity of 97% and 86% [85]. A more recent meta-analysis from 2011, only including studies on average risk individuals, found sensitivity of 83% and 76% for polyps ≥10mm and ≥6mm with corresponding specificity of 99% and 95% [86]. Bowel preparation for CTC is the same as for colonoscopy, but the examination is minimally invasive and takes just a few minutes including insufflation of air.

It is not settled which screening program is best. The ideal program has high sensitivity for malignant and premalignant lesions, high corresponding specificity and includes removal of premalignant lesions. Individuals should experience minimal harm and discomfort in order to ensure a high participation rate which is crucial to achieve a positive effect. Finally, the program must be cost effective and without negative impact on treatment of other patients.

A Norwegian pilot project of screening for colorectal cancer was implemented in 2012. The project is designed as a randomized study where participants are distributed between annually FOBT and one-time flexible sigmoidoscopy. The results from this study will guide the design of a future screening program in Norway. It is however likely that in the future, better non-invasive tests, as the multi-gene panels of aberrant methylation described above, will improve the effect of screening. In addition to higher sensitivity and specificity in the detection of cancer compared to FOBT, these tests will also identify individuals with premalignant lesions. Subsequent endoscopy with removal of polyps will reduce the risk of colorectal cancer in these individuals and contribute to lower incidence of colorectal cancer in the population.

INTRODUCTION

Symptoms and signs in colorectal cancer. In a population with no systematic screening, the diagnosis of colorectal cancer is made after the patient presents with symptoms. The most common symptoms for colorectal cancer are rectal bleeding (58%), abdominal pain (52%) and change in bowel habits (51%). "Change in bowel habits" includes changes in consistency and shape of stools, and frequency or difficulty of evacuation. The most common signs are occult bleeding (77%) leading to iron deficiency anemia (57%) and fatigue [87]. In patients with cancer, anemia in combination with anorexia, nausea, vomiting, abdominal pain or fatigue was associated with location proximal to the splenic flexure. Distal tumors were associated with rectal bleeding and altered stools in addition to diarrhea, mucus, or rectal pain [87].

The clinical challenge for physicians in primary care is that these symptoms and signs are far from specific for colorectal cancer. In a study from general practice in the Netherlands, 9/269 (3.3%) of patients presenting with rectal bleeding was diagnosed with cancer. The association between rectal bleeding and colorectal cancer was highly age-dependent and only 1/229 (0.4%) of the patients younger than 60 years was diagnosed with cancer, compared to 8/40 (20%) of the patients older than 60 years [88]. Different combinations of symptoms and clinical signs have been published to improve prediction of cancer, but the results are convergent [89].

Diagnostic and preoperative examinations. A patient suspected to have colorectal cancer should be referred to a complete colonoscopy. If a tumor is encountered, a biopsy should be performed to confirm the diagnosis. Any polyps should be removed and sent to histopathological examination. If a colonoscopy is not possible or successful, a CT colonography (CTC) can be performed instead. It does not give the opportunity of taking biopsies and remove polyps, and has lower sensitivity for detecting small or sessile polyps, but is equally sensitive for advanced adenomas and cancer [90].

If a probable cancer is identified, CT of the thorax and abdomen should be performed without awaiting the result of the biopsy to confirm tumor location, look for distant metastasis, and involvement of neighboring organs. Carcinoembryonic antigen (CEA) and standard preoperative screening analyses should be analyzed in blood.

In the case of rectal cancer, Magnetic resonance imaging (MRI) of the pelvis must be performed to estimate the shortest distance between the primary tumor or a metastatic lymph node to the mesorectal fascia, the circumferential resection margin (CRM). The MRI

examination also provides necessary information in the planning of surgery. If MRI cannot be performed, CT of the pelvis and rectal ultrasonography (US) should be performed. Rectal US is also the best imaging modality for discriminating between pre-malignant adenomas and early rectal cancers.

Treatment

Treatment of colon cancer without distant metastases. Surgery is the primary treatment in colon cancer and a necessity for cure. In the case of no distant metastases, surgery will be with a curative intent. The resection of the bowel wall should leave a minimum of 5 cm free margin to the tumor. The mesentery, including all lymph nodes and blood supply, should be resected at the level of the primary feeding artery ("high tie") [91].

If the histopathologic examination of the resected tissue detects metastasis to any of the mesenteric lymph nodes, the cancer is classified as stage III and the patient is recommended adjuvant chemotherapy if fit and up to (75) 80 years old. The purpose of the treatment is to eradicate microscopic disease and a central study from 1995 showed improved 3 years event free survival from 78% to 83% [92].

In Norway, standard adjuvant treatment up to 70 years is 5-Fluorouracil (5-FU) in combination with folinic acid (Leucovorin) and oxaliplatin (FLOX or FOLFOX4). For older patients, the combination of 5-FU and Leucovorin (Nordic FLV) is recommended. Both combinations are administered intravenously every other week for 6 months and should be initiated no later than 4-6 weeks after surgery (http://ngicg.no/handlingsprogram/nasjonale_handlingsprogrammer/). Peroral alternatives for 5-FU exist with equal effect, but with a different profile for adverse events, including higher levels of neurosensory toxicity [93].

Treatment of rectal cancer without distant metastases. A MRI is always performed in patients with rectal cancer. A positive CRM means that there is 1mm or less from tumor tissue to the mesorectal fascia and is the case in 10-20% of all rectal cancers. A positive CRM is associated with a local recurrence rate of 15-25% [94, 95]. The rate of local recurrence is significantly reduced after neoadjuvant (preoperative) radiation and chemotherapy [96]. Based on these and similar studies, the Norwegian guidelines recommend neoadjuvant treatment if the estimated CRM on MRI is ≤3mm. Resection of the tumor can be conducted 6-8 weeks after the last radiation session.

Total mesorectal excision (TME) is now standard procedure in rectal cancer. It implies that the tissue is divided in the plane of the mesorectal fascia from the peritoneal cavity to the sphincter apparatus. All draining lymph nodes will be included in the resection and the autonomic pelvic neural plexus is spared. When the tumor is located in the upper part of the rectum, partial mesorectal excision (PME) can be accepted if there is a minimum of 5 cm free margin distal of the tumor. The procedure of TME/PME in combination with neoadjuvant treatment has dramatically reduced the frequency of local recurrences and is one of the most important advances in gastrointestinal surgery during the last thirty years.

Treatment of metastatic or recurrent disease. Patients with synchronous metastases or recurrent local or distant disease should be treated individually after evaluation of a multidisciplinary team. Complete resection of metastases or recurrent disease can be possible for some patients, but the majority will be beyond cure and quality of life is the primary goal in further treatment of this group.

Acute presentation of colorectal cancer. Ten to thirty percent of colorectal cancers present as emergencies due to obstruction and/or bowel perforation, and occasionally due to excessive bleeding. Patients that present with acute disease are usually older, have more advanced disease and the tumor is typically located in the colon, not rectum. [97-99]. Emergency surgery is associated with a much higher risk of complications and mortality than elective surgery, and in the case of obstruction, efforts should be undertaken to decompress colon non-operatively. For left sided tumors, this can often be achieved by placement of an intraluminal stent, as “bridge to surgery” [100]. Resection can usually be performed after 1-2 weeks as an elective procedure on a stable and prepared patient. When an intraluminal stent or complete resection of the tumor is not possible a bypass or diverting stoma will be indicated. If there is bowel perforation into the peritoneal cavity immediate surgery is indicated, but primary anastomosis after resection is usually considered unsafe due to infection and a Hartmann’s procedure is undertaken. Complications connected to the stoma are however common and reversion of the stoma is often not conducted [101].

Staging

The first system for staging of colorectal cancer was presented by the pathologist Cuthbert E. Dukes at St Mark's Hospital in London in 1932 [102]. The staging was primarily for rectal cancers and did not include a stage for metastatic disease, but it formed the basis of the Dukes classification for colorectal cancer still in use today. In 1946, Pierre Denoix devised the TNM system for solid tumors, which is now regularly revised by the Union for International Cancer Control (UICC). There were many similarities between the two staging systems, but because of several revisions over the years, especially in the last edition of the TNM-Classification, direct comparison is challenging. This also complicates the comparison with the modified Aston-Coller staging system and the general rules from the Japanese Society for Cancer of the Colon and Rectum [103]. Here the focus will be on the TNM classification.

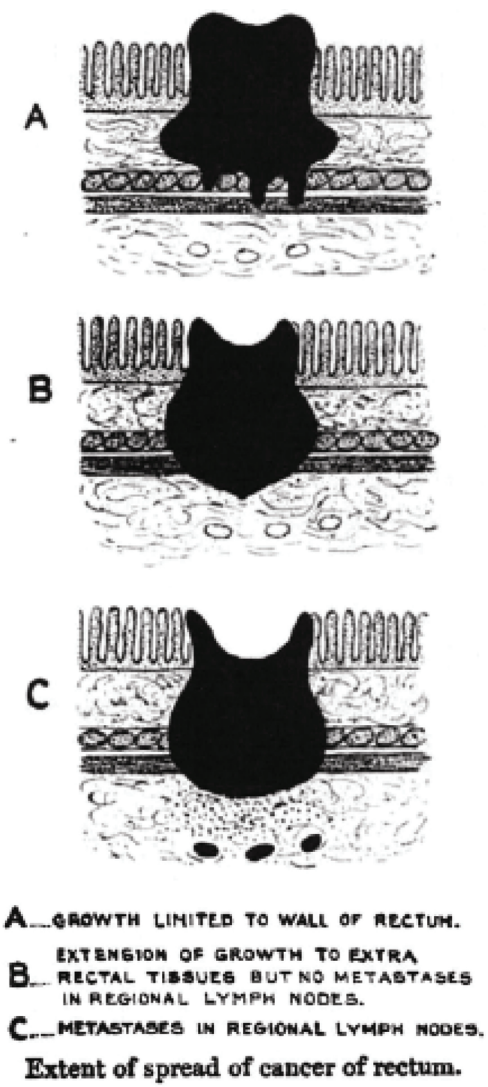

Illustration from Dukes paper in 1932

The "T" describes the extent of the primary tumor, "N" the number of metastatic regional lymph nodes, and the "M" refers to the presence of distant metastases. See Table 6 and Table 7 below for details in the classification in the 5th, 6th, and 7th edition of "TNM Classification of Malignant Tumors". The histopathological grading describes the differentiation of the tumor and is also included along with the R-classification that describes residual tumor after resection and relies on clinical, radiological, macroscopic and microscopic examinations of the patient and the resected tissue.

Table 6. The TNM classification.

TNM Clinical Classification	
T-stage	**Primary tumor**
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria.
T_1	Tumor invades submucosa
T_2	Tumor invades muscularis propria
T_3	Tumor invades through muscularis propria into pericolic or perirectal tissue.
T_4	Tumor invades other organs or structures and/or perforates visceral peritoneum
T_{4a} (7^{th} ed.)	Tumor penetrates visceral peritoneum
T_{4b} (7^{th} ed.)	Tumor invades or is adherent to other organs or structures
N-stage	**Metastatic regional lymph nodes**
N_0	No regional lymph node metastasis
N_1	Metastasis to 1-3 regional lymph nodes
N_2	Metastasis to ≥4 regional lymph nodes
N_{1a} (7^{th} ed.)	Metastasis to 1 regional lymph nodes
N_{1b} (7^{th} ed.)	Metastasis to 2-3 regional lymph nodes
N_{1c} (7^{th} ed.)	Tumor deposit in the pericolic/perirectal tissue
N_{2a} (7^{th} ed.)	Metastasis to 4-6 regional lymph nodes
N_{2b} (7^{th} ed.)	Metastasis to ≥7 regional lymph nodes
M-stage	**Distant metastasis**
M_0	No distant metastases
M_1	Distant metastases
M_{1a} (7^{th} ed.)	Metastasizing to only one organ or one site
M_{1b} (7^{th} ed.)	Metastasizing to multiple organs or peritoneal dissemination
Histopathological grading	
G-stage	**Grade of differentiation**
G_X	Grade of differentiation cannot be assessed
G_1	Well differentiated
G_2	Moderately differentiated
G_3	Poorly differentiated
G_4	Undifferentiated
R-Classification	
R-stage	**Residual tumor**
R_0	No residual tumor
R_1	Microscopic residual tumor (cancer cells ≤1mm from the res. margin)
R_2	Macroscopic residual tumor

Table 7. Outline of the different editions of "TNM Classification of Malignant Tumors"

	Edition		
TNM-stage	**5th (1997)**	**6th (2002)**	**7th (2009)**
I	T_{1-2}, N_0, M_0	T_{1-2}, N_0, M_0	T_{1-2}, N_0, M_0
II	T_{3-4}, N_0, M_0		
IIA		T_3, N_0, M_0	T_3, N_0, M_0
IIB		T_4, N_0, M_0	T_{4a}, N_0, M_0
IIC			T_{4b}, N_0, M_0
III	T_{1-4}, N_{1-2}, M_0		
IIIA		T_{1-2}, N_1, M_0	T_{1-2}, N_1, M_0 / T_1, N_{2a}, M_0
IIIB		T_{3-4}, N_1, M_0	T_{3-4a}, N_1, M_0 / T_{2-3}, N_{2a}, M_0 / T_{1-2}, N_{2b}, M_0
IIIC		T_{1-4}, N_2, M_0	T_{4a}, N_{2a}, M_0 / T_{3-4a}, N_{2b}, M_0 / T_{4b}, N_{1-2}, M_0
IV	T_{1-4}, N_{1-2}, M_1	T_{1-4}, N_{1-2}, M_1	
IVA			T_{1-4}, N_{1-2}, M_{1a}
IVB			T_{1-4}, N_{1-2}, M_{1b}

Morphologic and molecular development of colorectal cancer.

The polyp-cancer sequence was described by Muto in 1975. Based on a large series of adenomatous polyps and cancers from St Mark's Hospital he concluded that all colorectal cancer develops via polyps. Only a minority of polyps will progress to cancer, but the risk of malignant transformation was higher in polyps larger than 2 cm, with villous architecture or severe epithelial atypia. However, the development from normal mucosa to invasive cancer was slow and estimated to take from 5 years to a life time, with an average of 10-15 years [64]. Hyperplastic polyps were not included in the study since they were not regarded as preneoplastic lesions at the time.

The adenoma-carcinoma sequence. In 1990 Fearon and Vogelstein presented a model for the genetic basis of the polyp-cancer development and called it the adenoma-carcinoma sequence [104]. The model was based on inactivation of tumor suppressor genes and activation of oncogenes and how this was achieved through point mutations and loss and gain of chromosomal regions. Based on the reported frequency of allelic losses in different stages of adenomas and carcinomas, they indicated that a minimum of 4-5 genetic alterations were necessary for the transformation of a normal cell into a cancer cell and that the total accumulation of changes, rather than their order was responsible for the biological

properties of the tumor. They also discussed the dilemma with the recessive model of tumor suppressor genes. The model implies that both alleles must be inactivated for loss of function. However, if inactivation of one allele does not lead to a selection benefit, the probability of inactivation of the other allele is rather small. For mutation in *TP53*, a dominant negative function was described; the gene product from mutated *TP53* inactivates the wild type gene product by binding to it and thereby prevents normal association with other ligands [104]. Any reduction in normal p53 activity, including activation of DNA repair, growth arrest, and initiation of apoptosis will lead to a selection benefit.

In retrospect it is clear that the models presented by Muto and Fearon were far from complete. None discussed the possibility of precursor lesions to polyps/adenomas. Muto excluded hyperplastic polyps from his studies based on the assumption that they did not have any malignant potential and Fearon described a molecular model were chromosomal instability (CIN) are the only mechanism for carcinogenesis. Both assumptions have later been proven wrong, but the models gave an important framework for further studies and more comprehensive knowledge.

Aberrant crypt foci. Aberrant crypts in the colorectal mucosa were first described as possible early neoplastic lesions in 1984 [105]. An aberrant crypt has altered luminal opening, thick epithelial lining and is longer than a normal crypt in the colorectal mucosa. In 1987 Bird demonstrated that the number and size of aberrant crypts in mice increased after exposure to a colon carcinogen. Repeated exposure resulted in clusters of two or more aberrant crypts in so called aberrant crypt foci (ACF) [106].

By 1998, ACF was recognized as likely precursors of adenoma and cancer, but had mainly been studied in surgical specimens from patients with known colorectal cancer. In a study including 350 persons, Takayama used magnifying endoscopy to study number, size and dysplastic features of ACF in normal subjects, patients with adenomas and patients with colorectal cancer (Figure 3) [107]. Three patients were examined for ACF in the entire colon and rectum. Since 80% (9/11) of the detected ACF were located in the rectosigmoid and all three patients had at least one lesion in this area, examination in the rest of the patients was confined to the lower rectal area. Takayama found that the number and size increased with age and that there was a correlation between the number of ACF, the presence of dysplasia, size of the foci and number of adenomas. After treatment with a non-steroidal anti-inflammatory drug (NSAID) there was a significant decrease in the number of ACF implying that ACF is a reversible, dynamic change. Five percent of the ACF were dysplastic. Mutation in

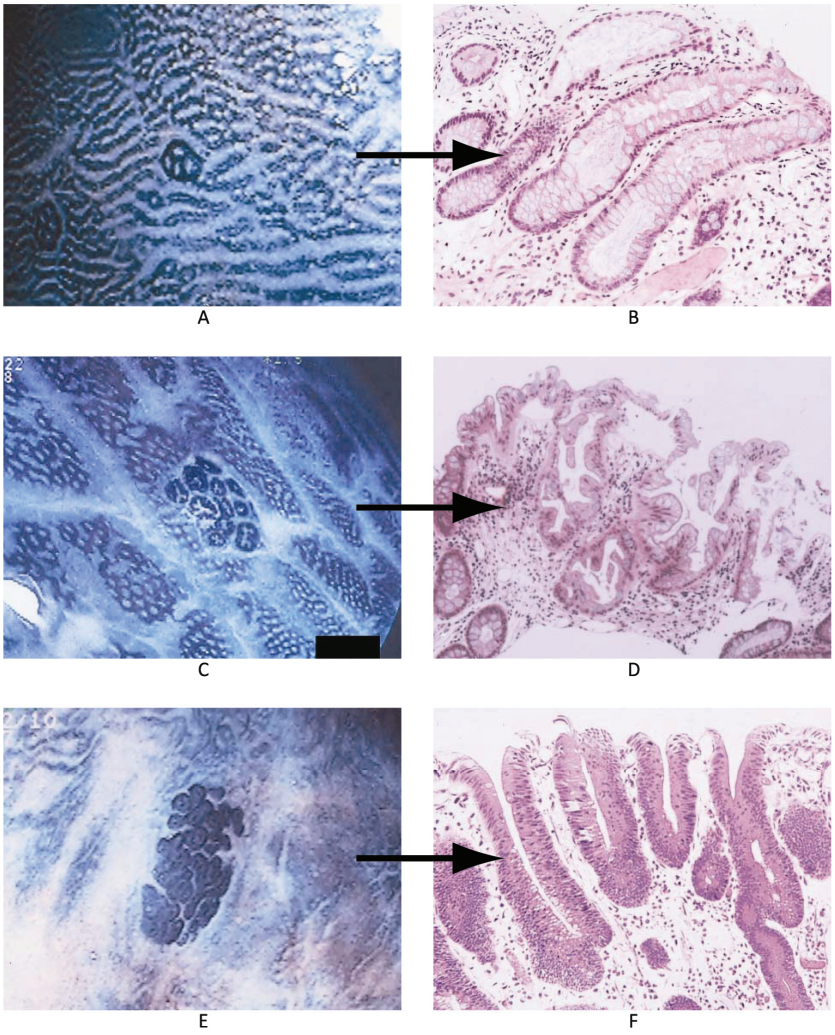

Figure 3. Endoscopic and Histological Features of Aberrant Crypt Foci. Panel A: Endoscopy with methylene blue staining reveals a small focus consisting of four crypts with semicircular or oval lumens. **Panel B:** Histologically, there was slight enlargement, irregularity, and elongation of the ducts, findings consistent with features of aberrant crypt foci without dysplasia or hyperplasia. **Panel C:** A medium focus consisting of 13 crypts, each with an asteroid or slit shape. **Panel D:** Histologically, there was a serrated luminal pattern, characteristic of aberrant crypt foci with hyperplasia. **Panel E:** A large focus with a deformed and slightly raised shape. The epithelial lining was thicker than those of the foci shown in Panels A and C, and each lumen was compressed or not distinct. **Panel F:** Histological examination revealed a loss of polarity, hyperchromatism of the nuclei, and stratification of the nuclei of crypt epithelium, findings in agreement with the previously reported features of dysplastic aberrant crypt foci. Endoscopy with methylene blue and histology with hematoxylin and eosin staining (x180 in B, x150 in D, and x120 in F). Reproduced with permission from [107], Copyright Massachusetts Medical Society. Text slightly modified.

KRAS codon 12 were found in 80-90% of heteroplastic ACF compared to 60% in dysplastic foci, suggesting that other genetic alterations are involved in the formation of dysplastic ACF [107].

In a recent review by Lopez-Ceron the role of ACF in carcinogenesis is confirmed and reinforced based on the common molecular changes observed in ACF and cancer. The reported prevalence of ACF is 15-77% in a healthy population and 80-100% in patients with colorectal cancer [108]. They are highly dynamic lesions, but studies exploring possible protective agents and factors, have had diverging results. [107, 109]. This might be related to the technically difficult endoscopic method, high variability in classification between pathologists and a possible difference in ACF-prevalence between different ethnic groups [108].

Aberrant crypt foci (ACF) can be classified as dysplastic or heteroplastic (including hyperplastic), [110]. The dysplastic ACF is characterized by abnormal epithelial proliferation in the luminal part of the crypt, lack of methylation and mutation in KRAS, and is regarded as the precursor of the traditional adenoma in the adenoma-carcinoma sequence. Dysplastic ACF is also the precursor of carcinogenesis in Familial Adenomatous Polyposis (FAP), but do then always carry a mutation in APC and seldom in KRAS [110].

For the heteroplastic ACF, two different pathways have been suggested. The non-serrated heteroplastic ACF are proposed as the precursors of the hyperplastic polyp in the distal colon and rectum. It is characterized by frequent mutations in KRAS and loss of 1p [111]. The serrated heteroplastic ACF often have mutation in BRAF and CpG Islands methylation (CIM) and is regarded as the precursor of the sessile serrated adenoma/polyp in the proximal colon and evolve along the serrated pathway.

The serrated pathway. For a long time, the hyperplastic polyp, ten times more common than the adenoma, was considered to be harmless without any malignant potential [112]. In 1990 Longacre and Fenoglio-Preiser described a series of 110 “mixed hyperplastic adenomatous polyps” (MHAP) with serrated morphology as in hyperplastic polyps but with cytological changes comparable to those observed in traditional adenomas [113]. Thirty seven percent of the MHAP demonstrated significant dysplasia, often at the basis of the crypts, while 10% also contained foci of intramucosal carcinoma. Based on the morphology, they concluded that the MHAP should be considered a distinct subtype of colorectal epithelial neoplasia and suggested the term “serrated adenoma”.

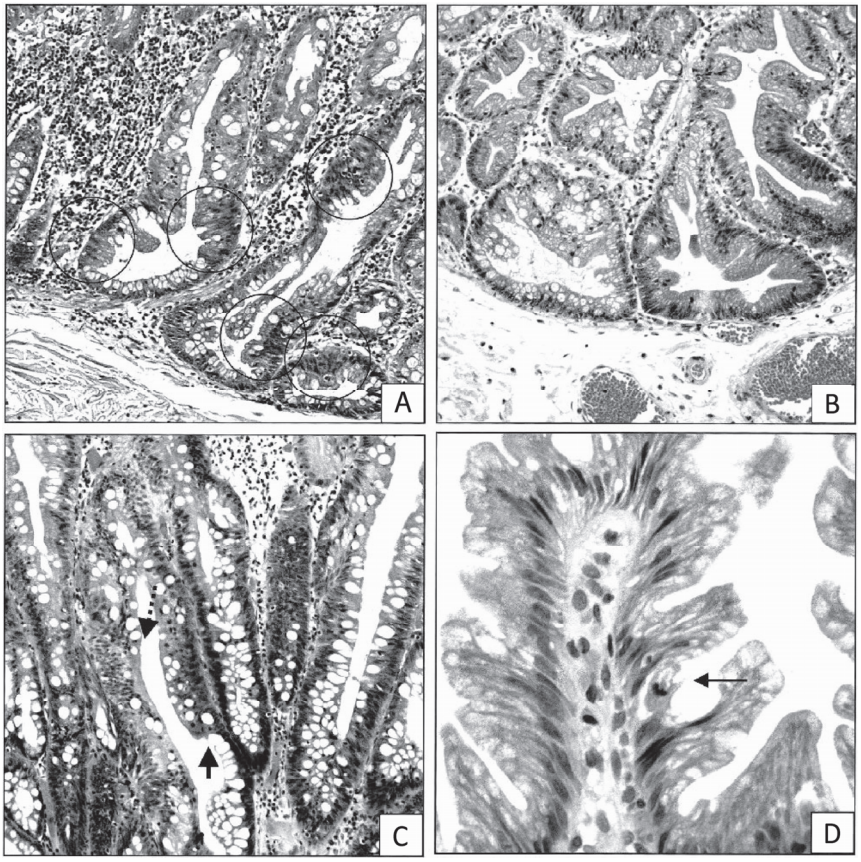

Figure 4. Serrated polyps with abnormal proliferation. Panel A: Irregularly distributed short segments of epithelium with lack of maturation (absence of mucin production), pseudo-stratification and an increased nucleus to cytoplasmic ratio (circled areas), are morphologic evidence of abnormal proliferation. Such segments are typically, seen luminal to segments of epithelium with normal proliferation. **Panel B:** The right dilated crypt has a proliferative appearance in the base, whereas the left dilated crypt does not. Asymmetry may also be observed in a single crypt. **Panel C:** Elongation, crowding, and stratification of the nuclei are present on the left side (dotted line arrow), whereas hypermature epithelium with goblet cells is seen only on the right side. In addition, proliferative epithelium is displaced toward the lumen (full line arrow). **Panel D:** The presence of mitoses close to the surface also indicates proliferation. Hematoxylin and eosin staining (×100 in A–C, ×400 in D). Reproduced with permission from [114], Copyright Lippincott Williams & Wilkins, Inc. Text slightly modified.

After the discovery of different morphological types within what was earlier regarded as a homogenous group of hyperplastic polyps, the research and knowledge in this field increased. In 2003, Torlakovic et al. evaluated 24 morphologic variables in 289 serrated polyps from colon and rectum, including proliferation (Figure 4). Among other, they found that almost 85% of serrated polyps were located in the distal colon and rectum, but that

Table 8. Molecular and clinical features of serrated lesions and suggestions for surveillance.

Features	Serrated lesion			
	Hyperplastic polyp	Sessile serrated adenoma/polyp		Traditional serrated adenoma
		No dysplasia	With dysplasia	
Molecular	**Frequency**			
CIMP-H	+[1]	+++	+++	++
MLH1-methylation	-	-	++	-
MSI	-	-	++	-
BRAF mutation	+[1]	+++	+++	+
KRAS mutation	+[2]	-	-	+
Clinical	**Frequency**			
Prevalence (%)	Very common	Common		Rare
Proportion of serrated lesions	70-95%	5-25%		<2%
Location	Distal colon, rectum	Proximal colon		Distal colon, rectum
Shape	Flat, sessile	Flat, sessile		Sessile or pedunculated
Size	Small, often <5mm	Larger than HP		Larger than HP
Precancerous	no	yes		yes
Surveillance	**Suggestions**			
Interval in years[3]	5-10	1-5	1-3	3-5

[1]MVHP commonly have CIMP-H and BRAF mutation
[2]GCHP commonly have KRAS mutation
[3]Depending on size, number and location of removed lesions.

polyps with abnormal proliferation were evenly distributed throughout the colorectum and often demonstrated loss of expression of MLH1 and/or MSH2 in the luminal part of the crypts. Based on their thorough morphologic evaluation, they proposed a classification

system for serrated polyps which is very similar to the system later given by the World Health Organization (WHO) [114].

In a consensus meeting in 2010, an expert panel met to summarize the field of serrated polyps and give recommendations for treatment [115]. They followed the WHO-classification dividing serrated lesions in three groups; Hyperplastic polyp (HP), Sessile serrated adenoma/polyp (SSA/P, with or without dysplasia), and Traditional serrated adenoma (TSA). The HP group can be subclassified as goblet cell type (GCHP), microvesicular type (MVHP), and mucin poor type (MPHP) based on the histological characteristics of lining epithelium. A consensus was reached for classification, clinical and histopathological features, and malignant potential of the different types. Strategies for improving endoscopic discovery and treatment were discussed and different surveillance regimes after resection of serrated lesions were suggested. The clinical and molecular features of the different groups of serrated lesions are displayed in Table 8 and a simplified model for colorectal cancer development is given in Figure 5.

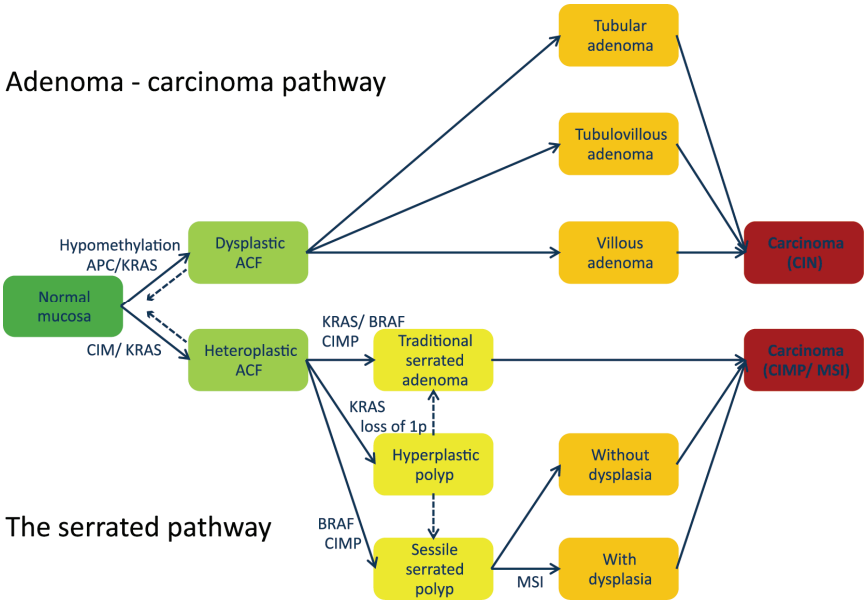

Figure 5. The morphological development of colorectal cancer.

Molecular phenotypes in sporadic colorectal cancer

The majority of colorectal cancers are sporadic and evolve in patients without any family history of cancer and thus without a germ-line mutation as the initiating event. Median age for diagnosis is 72 years [116], significantly older than for the hereditary syndromes. There are three recognized molecular phenotypes in sporadic colorectal cancer; microsatellite instability (MSI), chromosomal instability (CIN) and CpG island methylator phenotype (CIMP) [117, 118].

Microsatellite instability (MSI) is the best understood phenotype in colorectal cancer. It is caused by defect mismatch repair (MMR) and characterized by accumulation of errors in repetitive sequences called microsatellites throughout the cancer genome. A microsatellite is defined as a stretch of DNA made up of repeated sequences of nucleotides and is described according to the length and the number of repeats [119]. Microsatellites are abundant in the human genome and are usually located in non-coding regions, but can also be found in exons. They are highly polymorphic due to variation in repeat-length and are utilized in population genetics and forensic medicine.

Loire et al. analyzed all coding sequences of the 22 218 genes in the human genome to identify those with hypermutable repeats [120]. They found that 1 291 genes have a mononucleotide repeat of minimum 8 bases, 678 have a dinucleotide repeat of minimum 5 units, 39 have tetranucleotide repeats of 4 or more units, and 11 genes have pentanucleotide repeats with a minimum of 4 repeats. A total of 1 935 (8.7%) genes hold hypermutable repeats that are prone to insertions and deletions. The monorepeats are overrepresented in genes involved in cell cycle regulation and response to DNA damage stimuli [120], while the other repeats are evenly distributed across different functions. These hypermutable repeats are especially vulnerable for mutations in tumors with deficient MMR.

Lynch syndrome patients carry a germ line mutation in one of the MMR-genes, explaining the early onset of cancer. Sporadic MSI tumors are typically caused by epigenetic silencing of MLH1 due to hypermethylation of the promoter [30, 121]. The MMR system recognizes and repair insertions and deletions (indels) that occur during replication. Indels arise more often in microsatellites due to slippage during replication and lead to the forming of nicks and small loops in the double stranded DNA which are recognized by the MMR-complex [122].

The MMR is a highly conserved mechanism during evolution. The knowledge in the field is primarily based on research on the bacteria E. coli [25]. Several genes have been identified

which when mutated lead to defect mismatch repair and an accumulation of alterations in microsatellites. The gene products are therefore called “mut”-proteins. Three homodimers essential for mismatch repair in the E. coli has been identified; the MutS, MutL and MutH [123].

The eukaryote homologs of the MutS are the heterodimers MutSα and MutSβ while the MutL homologs are MutLα, MutLβ, and MutLγ. MutSα (MSH2+MSH6) recognizes single base-base mismatches and indels of 1-2 nucleotides while MutSβ (MSH2+MSH3) recognizes indels of ≥2 nucleotides. After binding to the mismatched DNA, they recruit MutLα (MLH1+PMS2) that introduces strand breaks and initiates repair by coordinating exonucleases and DNA polymerases (Figure 6). The roles of MutLβ and MutLγ are still unclear [124, 125].

An uncorrected insertion or deletion in the coding region of a gene usually results in a frame shift mutation, a premature termination codon, and loss of function of the protein. The tumor

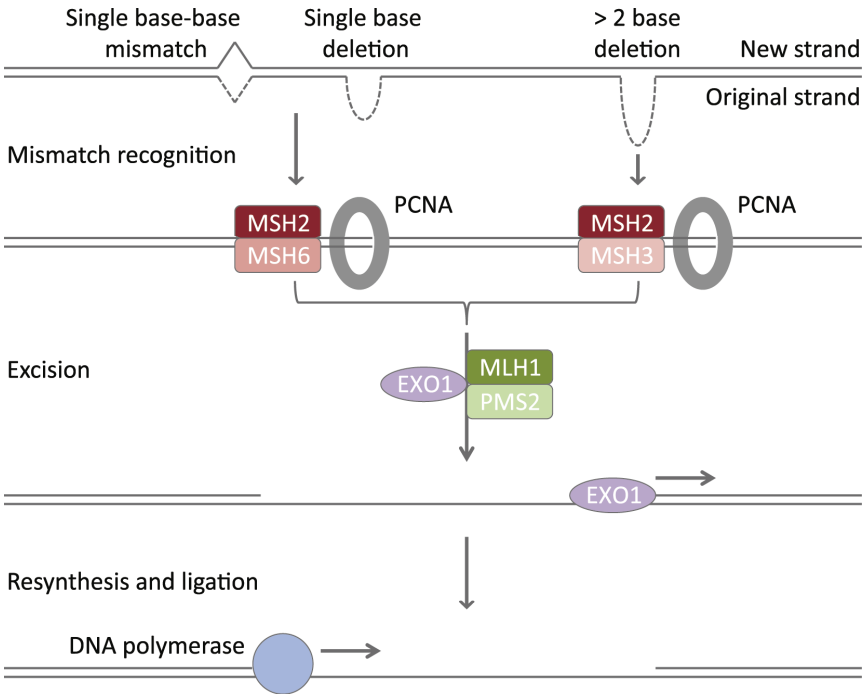

Figure 6. Outline of the mismatch repair system.

suppressor genes *TGFBR2*, *BAX*, *IGFR2*, *MSH3* and *MSH6* all carry microsatellites in their coding regions and represent typical target genes, commonly mutated in tumors with defect MMR [126, 127].

MSI was described in colorectal cancer by several authors in 1993 [20-23]. Tumors with MSI were found to be more common in the proximal colon, in women, and typically demonstrated poor or mucinous differentiation. A positive prognostic impact was indicated already in 1993 by Thibodeau and Lothe, respectively. However, the results from subsequent studies were diverging, possibly due to small or selected patient series. In 2005 a systematic review by Popat et al concluded that patients with colorectal cancer of the MSI phenotype indeed had better prognosis than those with MSS tumors [128]. A new systematic review and meta-analysis from 2010 confirmed this finding [129]. The prognostic effect in different stages and subgroups of patients has however not been settled.

The value of MSI in predicting benefit from treatment with 5-Fluorouracil (5FU) has also been explored. There have been contradictory results and a lack of controlled clinical trials where patients are randomized after stratification by MSI [130, 131]. However, a meta-analysis from 2009 found no difference in survival for patients with MSI tumors with or without adjuvant treatment and concluded that MSI is a negative predictive marker for benefit of 5FU based therapy [132].

Chromosomal instability (CIN) is the most common phenotype and is found in 65-70% of colorectal cancers. MSI and CIN are almost mutually exclusive, but a small overlap has been described [133]. The phenotype is not strictly defined but is characterized by an accumulation of structural and numerical chromosomal changes like translocations, deletions and amplifications resulting in copy number changes and/or aneuploidy (defined as any deviation from an exact multiple of the haploid number of chromosomes, whether fewer or more). Large deletions (more than half a chromosome arm) have been identified on chromosome 1, 4, 5, 8p, 14q, 15q, 17p, 18, 20p, and 21q while amplifications are typically observed on 7, 8q, 13q, 20 and X [134]. Areas that frequently experience loss often contain tumor suppressor genes, like *APC* (5q), *TP53* (17p), and *SMAD2/SMAD4* (18q). In addition to the chromosomal changes, point mutations in cancer critical genes are also observed.

CIN is observed in most solid tumors and is associated with poor prognosis and drug resistance due to intratumor heterogeneity. The basis for CIN is poorly understood, but suggested mechanisms include defect chromosome segregation, telomere dysfunction or

erroneous damage response [135]. A number of genes are involved in these mechanisms including *TP53* and *APC*, but none is yet identified as the initiator of CIN. The global DNA hypomethylation observed in many neoplasms, including colorectal cancer [136] might lead to reduced chromosome condensation and result in mitotic nondisjunction, and were suggested as a possible contributor to chromosomal instability by Fearon and Vogelstein [104]. Replication stress was recently introduced as another potential factor [137].

Several studies have demonstrated that patients with CIN tumors have worse prognosis than those with MSI tumors [138, 139].

CpG methylator phenotype (CIMP) is the third recognized phenotype in colorectal cancer. Methylation of nucleotides plays a central role in the regulation of DNA. It enables packing and condensation of the DNA molecule and can render a gene unavailable for transcription. In some colorectal cancers, hypermethylation at specific sites are observed, while at the same time a genome wide hypomethylation is observed with advancing disease stage.

In 1999, Toyota et al demonstrated that CpG Islands (see box) in a set of cancer specific genes were exclusively methylated in a subset of cancer cell lines and tumor samples and termed the phenomenon "CpG Island methylator phenotype" (CIMP). After observing these changes in both polyps and primary carcinomas, they concluded that this was an early event in colorectal carcinogenesis which was associated with transcriptional inactivation of tumor suppressor genes. They also found that CIMP-positive lesions were primarily located in the proximal colon [72]. CIMP has since gained approval as a defined pathway in colorectal carcinogenesis [140]. CIMP is now considered to be an early event and the major driving mechanism in the serrated pathway leading to the formation of the sessile serrated

The CpG dinucleotide and the definition of a CpG Island

A cytosine (C) followed by a guanine (G) at the same DNA strand, represents the most frequently methylated nucleotide in the genome. The CG dinucleotide is called CpG where "p" refers to the phosfodiester bound between the nucleotides.

A CpG Island are defined as a DNA region of at least 500bp where the C+G frequency is higher than 55% and the observed to expected CpG ratio is more than 65% [141]. About 40% of all human genes contain CpG Islands in their promoters and exonic regions [142].

adenoma/ polyp (SSA/P) in the proximal colon, the rare traditional serrated adenoma (TSA) in the distal colon and rectum, and eventually cancer [115]. The phenotype has been associated with older age, female gender, poor differentiation, BRAF mutation, MSI, and stable chromosomes [118, 143]. A negative prognostic impact of CIMP has been demonstrated, but only in MSS tumors [144, 145].

The methylation of CpG islands is a stable modification and is easily analyzed in formalin fixed tissue as well as in feces and blood. There is no agreement on a set panel of markers to define the phenotype, but several panels have been tested as biomarkers for early detection [75, 146], prognostication or prediction of treatment outcome [147]. The numerical relationships between the different phenotypes are illustrated in Figure 7.

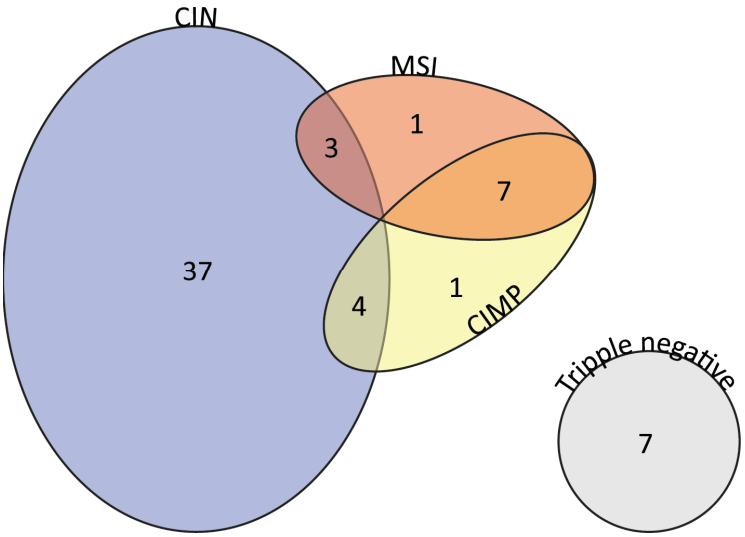

Figure 7. Venn diagram showing the relationships between the different molecular subtypes in colorectal cancer. CIN is here defined as aberration in chromosome 2p, 3p, 5q, 17p, and/or 18q [148]. Analyses were performed in a cohort of 60 patients and the numbers correspond to number of patients. Modified after Cheng et al. [118].

Prognostic factors

Prognosis (Greek. pro-gnosis, foreknowledge) -
a forecast as to the probable course and outcome of a disease

Prognostic markers are indicators of disease outcome and a large number of possible and definite prognostic markers have been identified for colorectal cancer. The best established and those considered in the original works of this thesis will be discussed here.

Clinical and histopathological factors

Age has negative prognostic impact in colorectal cancer. In analyses including death of any cause as an event, this should be expected, but even in analyses of relative survival and cancer specific mortality the oldest patients do worse [116]. This can be due to comorbidity resulting in less aggressive surgery and the age limit indicated in guidelines for adjuvant treatment can enhance this effect. Age should always be accounted for in prognostic analyses and included in multivariate models.

Gender had prognostic impact in a large Norwegian study were women had 12% lower relative risk of cause-specific mortality than men [116]. Others have also identified gender as a prognostic factor [149]. There is no recognized reason for this, but gender is associated with other prognostic factors like tumor location and MSI and should thus be accounted for in prognostic analyses.

Tumor location have demonstrated an independent prognostic impact in some studies [150], but most studies find location to be a non-significant marker [151]. It is however correlated to several other prognostic markers like age, tumor grade, MSI-status, BRAF-mutation, and urgency of surgery and should be accounted for in prognostic analyses.

Urgency of surgery. Between 9 and 35% of patients with colorectal cancers present as emergencies in need of acute surgery [99, 152]. They have higher postoperative mortality as could be expected, but also long term survival is inferior in this group [97, 98]. It can be argued that this is due to older age, advanced stage and postoperative mortality. However, a negative prognostic impact of acute surgery on long term survival has been demonstrated in several large series even after exclusion of postoperative deaths and controlling for stage [97, 153]. Mc Ardle also adjusted for age, sex, and tumor location with the same result [98].

A meta-analysis from 2011 evaluated the effect of self-expanding intraluminal stents as a bridge to surgery in patients with obstruction due to colorectal cancer. Even though the stent group required less intensive care, had higher rate of primary anastomosis, and fewer complications, it concluded that the long term survival did not improve [154].

Stage. The TNM classification for staging of colorectal cancer is described above. Stage is the strongest and best established predictor of outcome in colorectal cancer patients [149, 155]. It is however evident that with only four main stages, there is considerable heterogeneity within each stage when it comes to prognosis, especially for stage II and III [151, 156]. For better prognostication new subgroups within each stage can be introduced, as in the last version of the TNM-classification, or by identifying new biomarkers with prognostic impact. Any new marker should however be matched with stage to prove an independent prognostic value.

Histological grade and morphological subtypes. Histopathological grade is part of the TNM classification system discussed above and describes the differentiation of tumor as evaluated by histology. It is a well established prognostic marker and patients with high tumor grade (G3) have inferior prognosis to those with low or medium grade (G1 or G2) [149, 155, 157].

In addition to differentiation, the histological examination can identify various morphological variants. Two mucinous subtypes have demonstrated prognostic impact, signet-ring cell adenocarcinoma (SR) and mucinous adenocarcinoma (MAC). SR has intracellular mucin deposits while MAC is characterized by extracellular pools of mucin. Most studies demonstrate inferior prognosis, also after adjustments for stage, but a few studies show improved prognosis in tumors with MSI [158]. Kang et al describes the largest series, including over 160 000 patients from a national cancer registry, whereof 18 500 had one of the mucinous subtypes [159]. They found that both types were most common in the proximal colon, but only MAC was associated with female gender and only SR with advanced stage, poor differentiation, and inferior prognosis when adjusting for stage.

R-status describes residual tumor after surgery and is defined above in Table 6, page 32. It is recognized as a strong prognostic marker [157, 160]. Information about R-status is often missing in publications including patient series, but should always be accounted for in prognostic analyses.

Number of examined lymph nodes. Over the last decades, the total number of examined lymph nodes has been identified as an independent prognostic marker, both in node negative and node positive disease [161, 162].

Goldstein found that survival increased with the number of examined lymph nodes in stage II. The 5-year overall survival was 62% for those with <8 lymph nodes compared to 76% for those with >17 examined lymph nodes. Identification of metastatic nodes increased with the number of examined nodes and there was no minimum number for accurate staging [163]. In a study by Swanson et al, 5-year relative survival was 64% for patients were <3 lymph nodes was examined compared to 86% in those with >25 lymph nodes. They concluded that a minimum of 13 lymph nodes should be examined for correct N-staging [162]. In a systematic review from 2007 the association between the numbers of examined lymph nodes and prognosis was confirmed, both for stage II and stage III, but they did not suggest any number for adequate staging [164].

Lymph node ratio (LNR) is defined as the number of metastatic lymph nodes divided by the total number of examined lymph nodes. The LNR has prognostic impact in stage III colorectal cancer and metastases in a large proportion of examined lymph nodes lead to a high LNR and poor survival [165]. LNR is a stronger prognostic marker than the number of metastatic lymph nodes alone [166-168]. Berger et al showed that LNR had significant prognostic impact in colon cancer when ≥10 lymph nodes were examined. If less than 10 nodes were examined, the number of metastatic nodes had most impact [169]. In a systematic review from 2010 the LNR was confirmed to be a superior prognostic marker compared to the number of metastatic lymph nodes in stage III for colon and rectal cancer [170].

Micrometastases and isolated tumor cells in lymph nodes. Micrometastases (MM) are defined as clusters of tumor cells <2mm and isolated tumor cells (ITC) as solitary cells or clusters of cancer cells <0.2mm (TNM classification, ed. 6). These are difficult to identify by standard Hematoxylin and Eosin staining, but immunohistochemical staining by an antibody for cytokeratin [171-174] will expose occult cancer cells. A complete analysis involves cutting and immunohistochemistry of all isolated lymph nodes. PCR-based analyses are possible alternatives for identification of MM and ITC in lymph nodes.

The rate of microscopic disease in lymph nodes in stage I and II varies greatly between studies, but most series report a frequency of 20-50% [172]. Isolated tumor cells are reported to be most common in lymph nodes close to the tumor [175].

The identification of occult tumor cells in lymph nodes is associated with poor prognosis in some studies [173, 174, 176], while other studies fail to prove an effect [177, 178]. A systematic review and meta-analyses from 2012 included more than 4000 patients and concluded that MM and ITC in lymph nodes had negative prognostic impact in stage I and II [172].

Tumor invasion in venous or lymphatic vessels is a crucial step in hematologic and lymphatic tumor spread respectively. This can be detected in HE-stained sections, but specific staining for better identification of venous or lymphatic vessels reduce the rate of false negative and false positive, and helps distinguish between the two [173, 179, 180]. The reported incidence of venous and lymphatic invasion varies greatly, which can be due to differences in sectioning of the tumor, the number of examined tumor blocks, use of special stains, and interobserver variability [181].

Lymphatic invasion is associated with depth of tumor, poor differentiation, tumor budding, lymph node metastasis and stage [179]. Liang et al. found that patients with lymphatic tumor invasion have inferior prognosis in univariate analyses, but not when adjusting for stage and lymph node metastases [179]. Barresi et al. found a negative prognostic impact of lymphatic invasion, but also a significant association between lymphatic invasion and nodal MM. Considering the expensive and time consuming procedures connected to detection of MM by immunohistochemistry, they proposed the assessment of lymphatic invasion as a faster and cheaper procedure to identify patients with inferior prognosis [173].

Venous invasion is related to tumor depth and stage [179, 182]. It is an independent predictor of distal metastasis and inferior prognosis [179, 181]. In contrast to lymphatic vessels, veins have a basement membrane and elastin-staining will enhance the identification of veins and distinguish between lymphatic and venous tumor invasion [180].

Despite their potential as prognostic markers in a routine setting, no standard or guidelines for the pathological evaluation of lymphatic or venous invasion have yet been established [180].

Perineural invasion is less common than invasion of lymphatic and venous vessels, but has been reported in up to 33% of resected tumors [183]. It has been associated with stage and tumor grade, but an independent effect on prognosis in node negative diseases has also been

observed [184]. The results regarding prognostic value are however few and diverging [185-188] and no conclusion has been reached.

Lymphoid reaction in and around the tumor can be classified in four groups; Crohn's-like lymphoid reaction, peritumoral lymphocytic reaction, intratumoral periglandular reaction, and tumor-infiltrating lymphocytes. Combined, these showed an association with higher age, tumor location in proximal colon, high tumor grade, and MSI in a study by Ogino et al [189]. All types of lymphoid reaction were found to have a positive prognostic effect. However, in most studies a distinction between these departments of lymphoid reaction are not made and the lymphoid reaction is usually only described as tumor-infiltrating lymphocytes (TIL).

The prognostic impact of tumor infiltrating T-cell subsets has also been explored in several studies and a literature review by Nosho et al from 2010 recapitulated the results [190]. Most studies confirm a positive impact on prognosis for TIL, but there is diverging results concerning which subsets of T-cells that this applies to [191-193]. There are also differences in the criteria used in the histological evaluation. This must be settled before TIL can be introduced in the histopathological routine, but it has the potential to become an important prognostic marker in colorectal cancer [194].

Tumor budding refers to microscopic clusters of undifferentiated cancer cells ahead of the invasive border and was first described in Japanese studies in the 1950s [195]. It is sometimes referred to as the morphological manifestation of epithelial mesenchymal transition (EMT), a necessary step in the metastatic process [196]. The EMT involves several steps including loss of cell adhesion molecules, altered cytoskeleton, and resistance to apoptosis resulting in increased migratory and invasive capacity [197].

Tumor budding is associated with higher tumor grade, lymphovascular and perineural invasion, and lymph node metastasis, but has also independent negative prognostic impact in colorectal cancer stage I and II [197, 198]. In stage III the data is limited and diverging [199] and an effect would probably have less impact on clinical management.

The carcinoembryonic antigen (CEA) was identified as a tissue marker of malignant tumors of the endodermally derived epithelium of the gastrointestinal tract and pancreas by Gold and Freedman in 1965 [200]. It has later been demonstrated that preoperative elevated levels of CEA in serum is predictive of recurrence in colorectal cancer [201, 202]. The level of CEA in serum is associated with disease stage, tumor grade, liver disease, tumor location,

bowel obstruction, smoking and ploidy, but is proven to have an independent prognostic value in most studies [203]. There is however a lack of studies that show a benefit of postoperative treatment based on preoperative CEA levels alone and it is not included in guidelines for adjuvant treatment [204].

An elevated level of CEA should return to normal within 4-6 weeks after complete resection and subsequent elevation of CEA is indicative of tumor recurrence [205]. Hall et al. followed 149 patients after complete resection and found that all patients with relapse developed elevated serum CEA eventually and that CEA elevation preceded detection by other methods in 70% of the cases, by a median lead time of 5 months [206]. Early detection of relapse might allow for complete resection and CEA should therefore be regularly monitored postoperatively in patients that are candidates for resection of distant metastases [204].

Molecular markers

Microsatellite instability (MSI) was identified as a molecular phenotype in colorectal cancer in 1993 and a possible positive prognostic impact was documented in two separate reports the same year [20, 23]. Systematic reviews and meta-analyses have later confirmed MSI as an independent prognostic marker [128, 129].

KRAS is a proto-oncogene and a member of the ras family. The family is named after "rat sarcoma" because they were initially discovered in viruses causing sarcomas in rodents. Werner Kirsten was one of the researchers who identified the virus in the sixties, hence the "K" [207]. The gene product was identified as an intracellular polypeptide (p21) in 1977 and in 1984 demonstrated to be a small GTPase with impaired activity when mutated [208-210]. Mutated *KRAS* was identified in a biopsy from a lung cancer in 1984 [211] and has later been recognized as one of the most frequently mutated oncogenes in cancer. In colorectal cancer, activating mutations are present in 30-40% of tumors in population-based patient series [212-214]. *KRAS* mutations are considered an early genetic event and have even been detected in the majority of aberrant crypt foci [107].

The *KRAS* gene is located at the short arm of chromosome 12. The gene product is situated close to the cellular membrane and is activated by receptor tyrosine kinases like the epidermal growth factor receptor (EGFR) [215]. It activates BRAF [216] and contributes in the activation of PI3K (Figure 8) [217]. Mutations in the *RAS* genes are usually located in codon 12, 13, or 61 and lead to constant activation of the enzyme resulting in induction of a

wide range of downstream cellular processes as transcription and cell cycle progression [218].

The prognostic value of *KRAS* mutations is uncertain. The "Rascal II" study, a large multicenter study concluded that the glycine to valine mutation in codon 12 had negative prognostic impact [219]. One other large study has confirmed this finding [220], but most studies do not find any prognostic impact or have contradictory results.

In metastatic colorectal cancer, mutations in *KRAS* have been investigated and proven negative predictors of response to anti-EGFR treatment [221]. Anti-EGFR treatment in the form of Cetuximab or Panitumumab is now only recommended to patients with KRAS wild type tumors [222].

BRAF. The v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) is a proto-oncogene and member of the RAF kinase family [223]. It is activated by KRAS and regulates the mitogen-activated protein kinase (MAPK) cascade, including the extracellular-signal-regulated kinase (ERK) signaling pathway, which affects cell proliferation, cell-cycle arrest, terminal differentiation and apoptosis (Figure 8) [224, 225].

A mutation is detected in 10-15% of colorectal tumors [214, 226], the V600E substitution being the most common [227]. Mutations lead to constitutive activation and deregulation of the downstream signaling pathways. It is regarded an early event in the serrated pathway and has been identified in sessile serrated adenomas, but is mutually exclusive with mutations in *KRAS* [223].

Mutated *BRAF* has been identified as a possible prognostic marker in colorectal cancer, especially in microsatellite stable (MSS) tumors [228, 229], but the results are diverging to which clinical subgroups this applies [230, 231].

The predictive value of mutations in *BRAF* has been explored in advanced colorectal cancer in relation to anti-EGFR-treatment. Some find that tumors with *BRAF* mutation do not respond to treatment [232-234] while others do not find a significant predictive impact [235]. It is not yet settled if anti-EGFR treatment should be restricted to those with *BRAF* wt [236-238].

***PIK3CA*.** The class I Phosphatidylinositide 3-kinases (PI3K) function as heterodimers that phosphorylate inositol lipids. They are activated by receptor tyrosine kinases, and G-protein-

coupled receptors, including KRAS [239]. PI3K increase intracellular PIP_3 and activate AKT signaling, a complex network with a variety of actions (Figure 8) [240]. The heterodimers consist of a regulatory and a cathalytic subunit. *PIK3CA* encodes the catalytic subunit p110α that together with p85α makes one of the class IA PI3Ks.

PIK3CA is an oncogene and mutations in the gene are observed in a range of different cancers. Mutations are usually located at a few hotspots on exon 9 or 20 and lead to constitutional activation [241]. The mutation frequency is 10-15% in colorectal cancer and the prognostic value has been evaluated in several studies with diverging results [214, 242, 243].

The predictive value of *PIK3CA* mutation in relation to anti-EGFR treatment has been explored in some studies with diverging results [244-246].

PTEN. The phosphatase and tensin homologue (PTEN) acts as a tumor suppressor. It regulates cell activity by dephosphorylation of PIP_3, counteracting the effect of PI3K (Figure 8). The most common aberration in cancer is reduced expression of PTEN. The prognostic effect of loss of PTEN expression in colorectal cancer is not settled [247, 248], but several studies report an associated with resistance to anti-EGFR treatment [249-251].

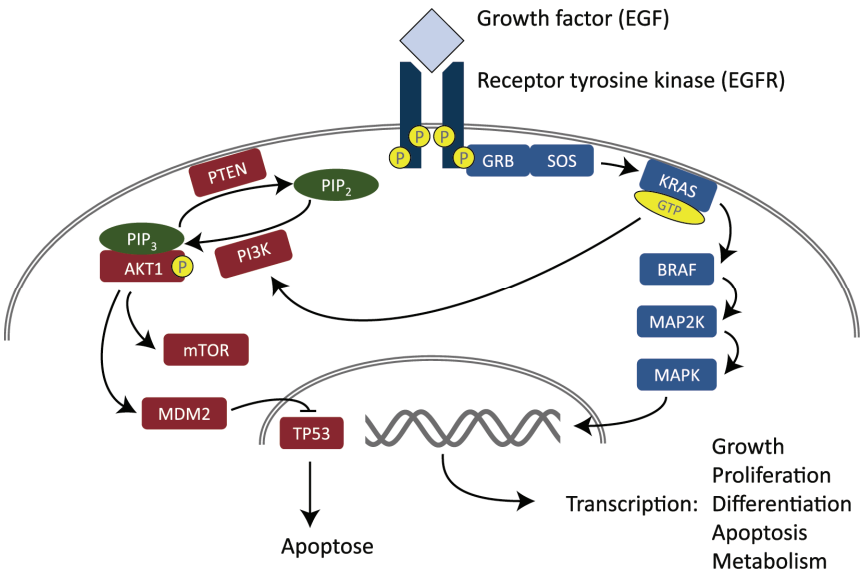

Figure 8. A simplified outline of the RAS-RAF-MAPK and PI3K signaling pathways.

Somatic mutations in two A_6 tracts in exon 7 and 8 of *PTEN* are reported in colorectal tumors with MSI [252]. The prognostic and predictive value of these mutations is not known.

Aneuploidy, where the nuclear DNA content deviate from a multiple of the haploid number of chromosomes, is regarded a result of chromosomal instability (CIN) [253]. It can be assessed by flow cytometry or image analyses [254, 255] and was first proposed as a prognostic marker in colorectal cancer in 1982 [256]. Several studies have been published since with diverging results [257-259]. However, two meta-analyses from 2007 and 2008 confirmed that tumors with aneuploidy have worse prognosis, but that the effect is confined to stage II and III [138, 260].

Loss of heterozygosity at 18q is one of the best investigated chromosomal alterations in colorectal cancer. It is also one of the most common, and was identified in 73% of carcinomas analyzed by Vogelstein et al. in 1988 [261]. It is caused by chromosomal instability and three tumor suppressor genes at 18q21 have been identified as possible targets for this deletion, *SMAD2/4* (Small Mothers Against Decapentaplegic homolog 2/4) and *DCC* (deleted in colorectal cancer). The observed allelic deletion has been investigated as a prognostic marker with diverging results [139, 262, 263]. A systematic review from 2005 found loss of 18q to be a prognostic marker [264], while other claim that the effect of LOH 18q is confounded by the association with CIN/MSS [265-267].

Gene expression signatures have emerged as a new method for prognostication in cancer over the last years and Oncotype DX is now accepted as a prognostic and predictive marker in early estrogen receptor positive breast cancer. In colorectal cancer, the only gene signature test available outside research settings is Oncotype DX Colon Cancer. It includes 12 markers and is based on a literature review and pre-selection of candidate genes which are evaluated in a retrospective test series and validated in independent series. The test assay is RT-PCR based, can be performed on formalin fixed, paraffin-embedded tissue, and stratifies patients within stage II into low, intermediate, and high risk of recurrence [268]. It is however not (yet) recommended for clinical use.

Another reported gene expression signature is the ColoPrint. It consists of a panel of 18 genes and is, in contrast to the Oncotype DX tests, based on genome-wide data from microarray analyses. The panel has independent prognostic potential in stage II and III and separates the patients into a low and a high risk group [269]. The array-based test requires fresh tissue and is not yet commercially available.

In 2012 two prognostic gene expression signatures were also published from our department, both based on genome wide microarray data and validation in independent patient series. The ColoGuideEx consists of 13 genes which stratify stage II colorectal patients into high and low risk groups [270]. The other signature is named ColoGuidePro and includes 7 genes. It identifies patients with colorectal cancer stage II-III with high and low risk of recurrence in both uni-and multivariate analyses. When patients are stratified according to stage, significant impact has only been validated in stage III [271]. For both ColoGuideEx and ColoGuidePro, RT-PCR based assays are under development.

AIMS

There is a need for better prognostication of patients with colorectal cancer, especially in stage II and stage III.

The aim of this project was to explore the prevalence and prognostic usefulness of readily available markers in a large, Norwegian, consecutive series of colorectal cancer.

MATERIAL AND METHODS

Material

The clinical database and tissue

In 1993, Professor Knut Nygaard at the department of surgery at Aker University Hospital initiated a registration of all patients admitted to the hospital with colorectal cancer. The motivation at the time was primarily quality control connected to the introduction of a new operating procedure for rectal cancer, total mesorectal excision (TME), and standardization of colorectal cancer surgery in general. The doctor discharging a patient with colorectal cancer was responsible for completing a detailed case record form with information about the patient, including results from diagnostic tests, urgency of surgery, type of resection, postoperative complications, and results from the histopathological examination. These prospectively collected data were entered in a local database.

Colon cancer patients younger than 76 years, and all rectal cancer patients who underwent curative surgery, entered a 5-year follow-up program. This included measurement of serum CEA every sixth months, radiological examination of the liver and lungs every sixth month for the first three years and thereafter yearly. For rectal cancer patients who had undergone a low anterior resection, proctoscopy was performed at same intervals as the radiological examinations, and all patients had a concluding colonoscopy after five years. At every postoperative check-up, a simple form was completed, with the date and whether any relapse was detected, and the data entered in the database. The inclusion of patients has been controlled against the Cancer registry of Norway, which receive notice of every patients diagnosed with cancer from all pathology departments in Norway. All registration in the database has been done under supervision by Professor Nygaards successor, Professor Arild Nesbakken.

From 2005, a consecutive series of fresh tissue has been collected from patients that undergo elective resection of colorectal cancer. Feces and blood have also been included in the biobank. The registration of clinical data and collection of tissue have continued after the Department of Gastrointestinal Surgery at Aker University Hospital was merged with the corresponding department at Ullevål University Hospital in 2012 as a part of the reorganizing of Oslo University Hospital. After 20 years, a total of approximately 2500

patients are now registered and the clinical data and fresh tissue have been included in several PhDs.

In 2005, patients who had undergone a major resection between 1993 and 2003 were identified for inclusion in the molecular analyses of the current thesis. Through the records and reports from the department of pathology, the best suited blocks of formalin fixed paraffin embedded tissue were identified and retrieved from the archives. The corresponding HE sections were re-assessed by a pathologist to confirm cancer and to mark the most representative area of the tumor and this area was sectioned for further analyses. Three copies of a tissue microarray (TMA) were also constructed, including all retrieved samples. Only a few tumor samples were not available, resulting in a true consecutive series.

This thesis includes clinical data from 1993 to 2009, and the next paragraphs explain central definitions and expressions used in this work.

Major resection. Only patients who underwent major resection were included in the present analyses. Major resection is defined as surgical removal of the tumor bearing bowel segment including the mesentery with the lymphovascular pedicle. This is standard procedure when the intention is to cure the patient and includes dissection of the mesentery with all regional lymph nodes and central ligation of the vessels, so called "high tie" (corresponding to D3 in Japanese nomenclature) [91]. This is now the standard in our department, but routines and surgeons have varied during the inclusion period and some patients might have had less extensive dissections (D2).

Tumor location. The colorectal continuum is divided into three separate locations, based on embryological development and clinical considerations. The proximal part of colon, including the first two thirds of the transverse colon, originates from the midgut while the distal colon and rectum develop from the hindgut. The blood supply follows these two departments as the superior mesentery artery supplies the foregut-derived colon and the inferior mesentery artery supplies the part developed from the hindgut. Proximal colon is therefore here defined as coecum through the transverse colon and the distal colon as the splenic flexure through the rectosigmoid flexure. Rectum is confined to 15 cm above the anal verge measured on a stiff proctoscope. It is located retroperitoneally in the pelvis with somewhat limited access and in close vicinity to nerves controlling the bladder and sexual functions, prostate or female genitals. The surgical treatment of rectal cancer therefore implies anatomically, technically and functionally challenges and these patients are considered as a separate group.

Molecular alterations in the tumor vary with tumor location. This can be due to the embryonic origin, but changes in the biochemical composition, consistency, or passage time of feces in different parts of the gut may also be of importance.

Synchronous tumors. If two or more colorectal adenocarcinomas were documented at the time of major resection, the patient was registered with synchronous tumors. Synchronous tumors can be an indication of genetic predisposition and is included in the clinical criteria of the Bethesda Guidelines [32]. Our objective was to explore prognostic markers in sporadic colorectal cancer and these patients were therefore excluded from our analyses. It also would have been challenging to decide which of the synchronous tumors that was clinically most relevant and should be included in prognostic analyses of the different biomarkers.

Staging and tumor grade followed the 5th edition of the TNM classification described above. In paper 2 and 3, well (G1) and moderately (G2) differentiated tumors were grouped together in all analyses. In paper 3, poorly (G3) differentiated and mucinous tumors were grouped in the multivariate analyses due to small numbers.

R-status was applied as described above. To specify: in our study a resection was also classified as R2 if the primary tumor was completely removed with free margins, but there was evidence of metastatic disease at radiological or clinical examination. If synchronous metastases could be completely removed in the same procedure as the primary tumor or shortly after, the sum of resections was classified as R0.

In analyses of frequency of clinical or biological markers, all solitary tumors from major resections were included, but in the prognostic analyses only R0 resections were assessed. It is reasonable to suspect that prognosis for patients with residual cancer depends mostly on load and location of residual tumor and that the biological changes in the primary tumor have less impact. Tumors from R1 and R2 resections were therefore excluded from the prognostic analyses.

Postoperative mortality was defined as death within 3 months after surgery. Only patients with R0 resection were included in the prognostic analyses and deaths within 3 months were therefore presumed not to be related to tumor biology, but rather caused by postoperative complications or comorbidity. Review of the clinical journals and the registered causes of death confirmed this, and patients who died within three months were excluded from the prognostic analyses.

Urgency of surgery. Most patients underwent tumor resection as a planned procedure. They were admitted the day before surgery and had routinely preoperative bowel emptying. However, based on the documentation of the procedures, fifteen percent had tumor resection as an emergency procedure due to bowel obstruction, perforation or life-threatening bleeding. Emergency of surgery has impact on prognosis both in the short and long term and is therefore included as an independent variable in our prognostic analyses.

Examination of lymph nodes. The number of examined lymph nodes and lymph node ratio in our series is based on the routine examination of the resected tissue. After 3-5 days of fixation in formalin, the specimens were searched for macroscopically evident lymph nodes and one HE-section of each lymph node underwent microscopic evaluation.

Methods

Literature review

For the third paper, we first summarized the current knowledge about prognostic and predictive value of mutations in *KRAS*, *BRAF*, *PIK3CA* and *PTEN*. A Medline search was conducted mid-November 2012. Including the terms (Colorectal neoplasm [MesH]) AND (KRAS, BRAF, PIK3CA OR PTEN [MesH OR keywords]) AND (Prognosis OR Survival OR Antineoplastic Agent [MesH OR keywords]) the search obtained 1090 references after filtering for English language, Human, and Abstract. Based on the title and abstract, all references were classified according to type of study and which markers that were discussed. If tumor samples from patients were analyzed, the number of included patients, tumor location, and tumor stage were registered. Finally, it was indicated if the prognostic or predictive value was calculated.

Of the 1090 identified studies, 332 had analyzed any of the genes of interest and conducted prognostic analyses in patient series. After excluding series including less than 300 patients or only stage IV, 80 studies remained. Forty two were excluded based on the complete paper due to overlapping patient cohorts and another 13 because information on mutation status was available in less than 300 patients or the prognostic value of the mutation was missing. In the end we identified 25 studies that included series of more than 300 patients in mutation analyses of *KRAS*, *BRAF*, *PIK3CA,* or the repeats in *PTEN,* and calculated the prognostic value. A supplementary search was conducted in October 21013 which identified four more studies

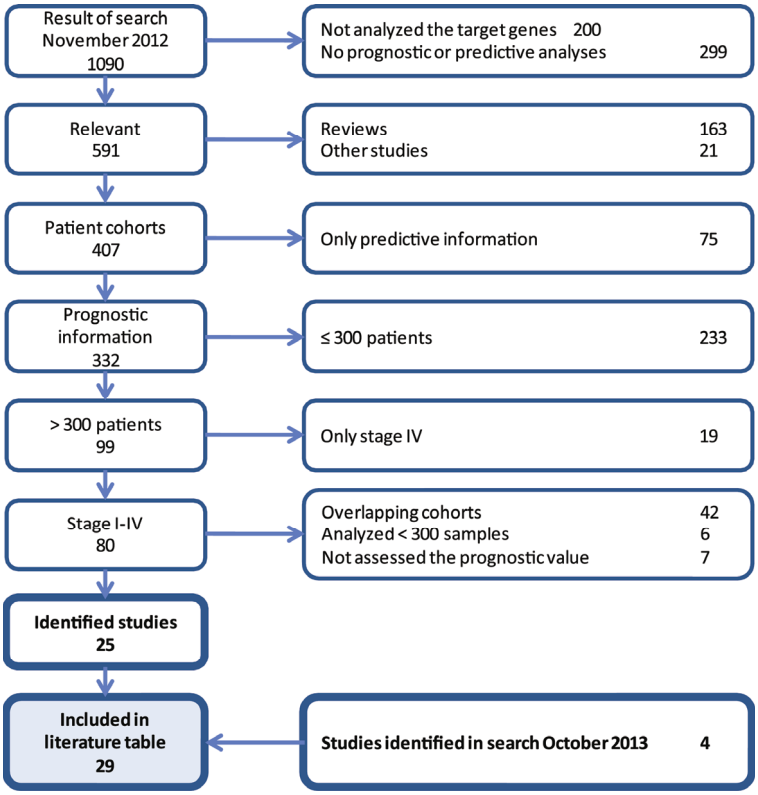

Figure 9. Summary of literature review and selection of papers in literature table.

that fulfilled the above criteria (**Figure 9**). These were presented in a table along with the main conclusions (see Paper 3). Nineteen of these studies have analyzed the prognostic impact of mutations in *KRAS* of which 8 document a significant effect. The results are however diverging when it comes to which specific mutations and groups of patients these this applies to. *BRAF* is analyzed in 18 studies and a significant prognostic impact is found in 9 whereof 4 describe a connection to MSI-status. *PIK3CA* is analyzed in only 3 of these large series with diverging results while no series at this size had analyzed the prognostic impact of mutations in the *PTEN* repeats.

DNA extraction

Four 25 µm sections were used for DNA extraction with the QIAamp DNA Mini kit from Qiagen. After buffer was added in the first step, a step of heating to 90°C for ten minutes for removal of paraffin was added. The rest of the procedure followed standard protocol. The

yield varied between different samples as expected, but were usually more than adequate. A PCR solution of 100ng/ml was made from the final eluate. While this was kept refrigerated the rest of the stock was divided and frozen.

Analyses of microsatellite instability (MSI)

For determination of MSI-status we conducted PCR-based fragment analyses of the five markers recommended in the Bethesda Guidelines from 1998 [33]. The two mononucleotide markers BAT25 and BAT26 were analyzed in the same reaction as were the three dinucleotide markers D2S123, D5S346 and D17S250. Both reactions used 37ng DNA template in a 10µl reaction volume (Multiplex PCR Master mix, fluorescent primers and water). The mononucleotide markers underwent 30 cycles and the dinucleotide markers 35 cycles. Fragment analyses were accomplished on the 3730 Genetic Analyzer (Applied biosystems) and DNA from blood samples from four healthy donors were included in every run as separate controls. The results were processed using the software GeneMapper (Figure 10) (Applied biosystems) and scored by two independent observers. Due to formalin fixation of the tissue, DNA was fragmented and quality was sometimes poor. Some samples were therefore run up to six times to achieve assessable results.

MSI-status for each of the markers was determined after two separate runs with the same conclusion. When poor DNA quality made scoring impossible or we found contradictory results, the marker was scored as "not determined". For each sample, MSI in two or more markers led to the conclusion of microsatellite instability-high (MSI-H). When one locus was MSI and four were MSS, the conclusion was microsatellite instability low (MSI-L). When all five markers were normal without sign of instability, the sample was scored microsatellite stable (MSS). In all statistical analyses, samples with MSI-L were included in the MSS group, as were samples were only four markers were assessable, but all were MSS.

Mutation analyses in *KRAS*, *BRAF*, *PIK3CA*, and *PTEN*.

Acknowledged hotspots in *KRAS*, *BRAF* and *PIK3CA* were analyzed. Since our aim was to identify clinically relevant mutations and the analyses were performed on DNA from FFPE tissue, a search for rare or new mutations outside these hotspots was not pursued. Exon 2 and 3 (codon 12, 13, and 61) were analyzed in *KRAS* while exon 15 (codon 600) was analyzed in *BRAF*. These hotspots represent more than 95% of all reported mutations in these genes in the "Catalogue of somatic mutations in cancer" - the COSMIC database - from Trust Sanger Institute (http://cancer.sanger.ac.uk/cancergenome/ projects/cosmic/).

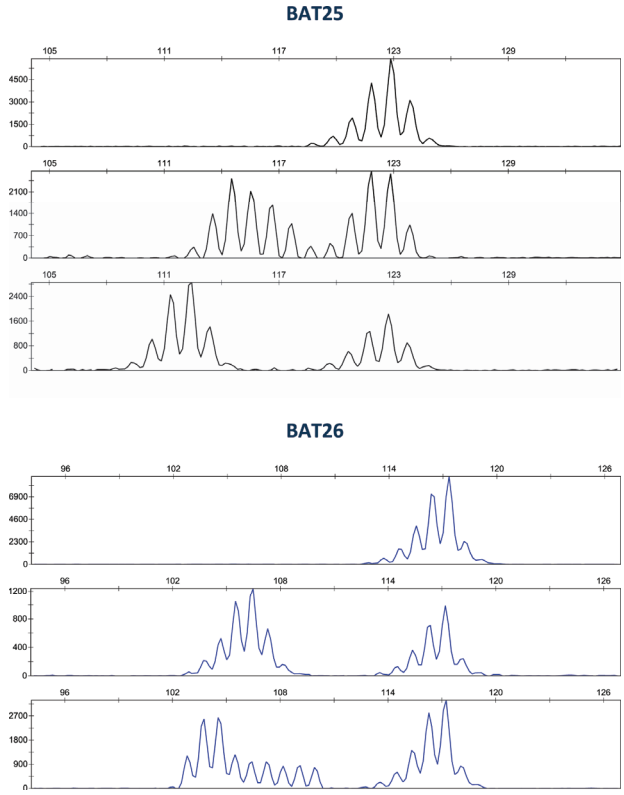

Figure 10. Fragment analyses of BAT 25 and BAT 26. Panels showing fragment analyses of BAT 25 and BAT 26. The first panel for each marker show normal fragment length (MSS), while the lower two panels show reduced fragment lengths corresponding to deletion of 7-14 bases (MSI).

In *PIK3CA* the hotspots are not as obvious, but there is an accumulation of mutations in exon 9 (codon 542 and 545) and exon 20 (codon 1025 and 1047). These mutations account for 70% of the reported mutations in COSMIC and we chose to limit our analyses to these codons since they represent the most probable hotspots in *PIK3CA*. In the MSI-tumors, three mononucleotide repeats in *PTEN* were also analyzed, the A_6 repeat in exon 7, and the A_5 and A_6 repeats in exon 8.

DNA was amplified using the Quiagen 2x Multiplex PCR-kit (Quiagen Inc, Valencia, CA, US). *BRAF* exon 15, *KRAS* exon 3 and *PIK3CA* exon 9 and 20 were amplified simultaneously, as were *KRAS* exon 2 and *PTEN* exon 7 and 8. The PCR products were purified enzymatically by ExoStar 1-step (VWR International Ltd, Leicestershire, UK) prior to sequencing reaction with Big Dye Terminator v1.1 Cycle Sequencing Kit for incorporation of dye labeled ddNTP's. The

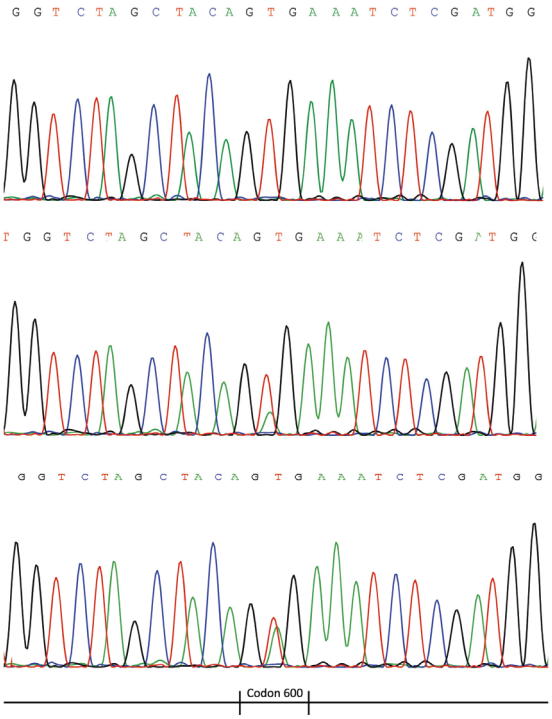

Figure 11. V600E mutations in the *BRAF* gene. Three panels showing the sequences of codon 596 to 604 in *BRAF.* The first panel shows a normal sequence while the second and third panel demonstrate a mutation in codon 600. **T** (Thymine) is replaced by a **A** (Adenine) at position 1799 (**T**1799**A**) resulting in a change of amino acid from Valine (V) to Glutamic acid (E) at this codon (V600E).

sequencing reaction was purified with Big Dye Xterminator (Applied Biosystems, Carlsbad, CA, US) and sequence analyses were performed on ABI 3730 DNA Sequencer (Applied Biosystems, Carlsbad, CA, US). The results were processed using the Sequencing Analysis 5.3.1 software (Figure 11) (Applied Biosystems). All results were scored independently by two observers and all mutations were confirmed in a second independent run. Some samples were run as simpleplex when necessary to obtain evaluable results.

Statistics

Endpoints. The decision on appropriate endpoints in the survival analyses was based on a review of available data in the clinical database. There had been routinely registration of local recurrence and distant metastases as well as date for the last clinical evaluation and death. Second primary colorectal cancer or other cancers had not been routinely registered.

Regarding cause of death, it was only indicated if the patient died of colorectal cancer or not. For patients who died in the hospital this information was obtained from the hospital journal and for those who died outside the hospital, information about cause of death was acquired from the Norwegian Tax Administration.

Autopsy is only performed in 10% of deaths in Norway implying that cause of death in 90% of the cases is based on the evaluation of the doctor confirming death and responsible for completing the death certificate [272]. If the doctor has treated the patient during the last weeks, the cause of death can be expected to be accurate. However, sometimes the doctor has limited knowledge about the patients, and cause of death is based on a brief review of available information from journals, nursing staff and relatives. Studies have questioned if the quality of the death certificates is adequate for research purposes [273].

Based on the available data and considerations around cause of death, we chose to use Overall Survival (OS) as the primary endpoint in all three papers. Death of any cause was registered as event and patients were censored at loss to follow-up defined as the last date of inquiry about death. In paper I, Time To Recurrence (TTR) was chosen as the second endpoint. Local recurrence, distant metastasis and death of same cancer were registered as events while patients were censored at death of other causes and loss to follow-up defined as last clinical evaluation. Relapse Free Survival (RFS) was chosen as the second endpoint in paper II and III. Documented relapse and death of any cause was registered as events while patients were censored at loss to follow-up defined as last date of clinical or radiological evaluation. Most relapses and cancer-related deaths in colorectal cancer occur within the first 3-5 years after diagnosis and primary treatment. Deaths more than five years after the primary treatment will to a lesser extent be related to the colorectal cancer and remaining patients were therefore censored at five years in the prognostic analyses.

OS, RFS and TTR are among the endpoints recommended by Punt et al after a systematic review of the use of endpoints in clinical trials for colorectal cancer [274].

Statistical calculations and tests. The association between categorical variables was explored in contingency tables. Pearson's Chi-square test was used to test independency of distribution, but Fischer's exact test was applied when the expected number in any cell was less than five and the total number in the table was less than 40. Continuous data (age, number of lymph nodes) had skewed distribution. Measures of central tendency were given as median (range) and t-test was applied for comparison of means. In the multivariate

analyses of impact on MSI-status and the number of examined lymph nodes, logistic regression models and Wald statistics were used. In prognostic univariate analyses, the Kaplan-Meyer method was used and survival distribution was compared with the Log-rank test while Cox regression models were applied in the multivariate analyses.

REMARK. The studies presented in this thesis meet the recommendations for tumor marker prognostic studies (REMARK) proposed by a working group formed at the NCI-EORTC First International Meeting on Cancer Diagnostics that was convened in Nyborg, Denmark in 2000 [275]. The recommendations were developed to facilitate evaluation of study design, methods, statistical analyses, and to improve the ability to compare results across studies.

RESULTS IN BRIEF

Paper I:

Prognostic Impact of Lymph Node Harvest and Lymph Node Ratio in Patients with Colon Cancer.

Sjo, Merok et al., Diseases of the colon & rectum (2012)

The prognostic impact of the number of analyzed lymph nodes (LN) and lymph node ratio (LNR) were explored in a series of 950 consecutive patients from Aker University Hospital. All patients who underwent a complete (R0) major resection for colon cancer stage I-III between January 1993 and December 2009 were included. Information of the number of LN and their metastatic status were based on routine histopathological examination and prospectively registered.

The study period were divided in three; 1993-1998, 1999-2004, and 2005-2009. There was a significant increase in examined LN (7 vs. 15, $p< 0.001$), the proportion of patients with ≥12 LN (18% vs. 85%, $p< 0.001$), and a non-significant increase in the proportion of stage III from the first to the last period (25% vs. 32%, $p=0.08$). Five year Overall Survival (OS) for all admitted patients (n=1481) improved during the study period (39% vs. 46%, $p=0.002$), but for the study cohort (I-III, R0) no improvement in OS was identified. Stage I and II had a significant improvement in time to recurrence (TTR) during the study period (81% vs. 95%, $p=0.02$ and 66% vs. 85%, $p=0.003$) but there was only a trend in stage III, when analyzed separately.

Patients were grouped according to the number of examined LN; <8 LN, 8-11 LN and ≥12 LN. The proportion of stage III was significantly associated with these groups (22%, 35%, and 33%, $p=0.001$). The number of examined LN had significant impact on OS and TTR in stage II, but only on TTR in stage III. In a Cox regression multivariate analysis, the number of examined LN had significant impact on both OS and TTR.

In stage III the lymph node ratio (LNR) was calculated and grouped according to quartiles; 0-0.10, 0.11-0.18, 0.19-0.40, and ≥0.41. The number of examined LN increased significantly

during the study period and the LNR decreased; the proportion of patients with the lowest LNR (0-0.10) increased from 9% to 33% from the first to the last time period. A higher LNR was significantly associated with reduced OS and TTR in both uni-and multivariate analyses.

Conclusion:
Overall survival for all patients with colon cancer improved in the study period (1993-2009) and a significant improvement in TTR was observed for stage I and II separately. The number of examined lymph nodes increased during the period and was associated with stage migration and improved OS and TTR. In stage III LNR is a stronger prognostic marker than the number of examined LN.

Paper II:

Microsatellite instability has a positive prognostic impact on stage II colorectal cancer after complete resection: results from a large, consecutive Norwegian series

Merok et al., Annals of oncology (2013)

Between January 1993 and August 2003, 925 consecutive patients underwent major resection of a solitary colorectal cancer at Aker University Hospital. Formalin fixed paraffin embedded tumor tissue were retrieved from the archive and microsatellite instability (MSI) was assessed by PCR based fragment analysis of the five loci recommended by the National Cancer Institute. A final conclusion was reached for 805 samples. Information about examined lymph nodes was based on the routine histopathological examination of the resected tissue and had been prospectively collected along with comprehensive clinical and pathological data.

MSI was significantly associated with female gender, proximal tumor location, tumor stage and high tumor grade in the univariate analyses, and with and female gender, proximal tumor location, high tumor grade and acute surgery in multivariate logistic regression analyses.

MSI had significant impact in both univariate and multivariate analyses of 5 year relapse free survival (5yRFS), but the effect was confined to stage II when analyzed separately. MSI had no prognostic effect in stage III, and in stage I and IV the numbers of MSI tumors were too small for any sound calculations.

For colon cancer the number of examined lymph nodes (LN) was categorized into two groups; <12 LN or ≥12 LN. The proportion of ≥12 examined lymph nodes was significantly associated with MSI-status, sex, age, tumor location and stage in univariate analyses, whereas age, tumor location and stage III were associated variables in the multivariate analysis.

Conclusion:
MSI is a marker for better prognosis in stage II colorectal cancer. The number of examined lymph nodes is significantly associated with age, tumor location, and stage, but not MSI in multivariate analyses.

Paper III:

BRAF-mutation has negative prognostic impact in microsatellite stable colon cancer stage II

Merok et al., manuscript

From the same patient series as described in paper II, 885 tumor samples from major resections of solitary colorectal cancers were included in analyses of recognized hotspots for mutations in *KRAS*, *BRAF*, and *PIK3CA*, whereas only the microsatellite instable (MSI) tumors were included in analyses of three microsatellite loci within exon 7 and 8 of *PTEN*. Mutation-status was assessed by PCR and direct sequencing.

The prevalence of mutations in *KRAS* and *BRAF* were in accordance with published data while the frequency of mutations in *PIK3CA* was similar to another series from the same geographic area, but lower than described in non-Norwegian series. The frequency of *PTEN* mutations in MSI tumors were close to the suggested cut-off for passenger mutations in MSI tumors.

RESULTS IN BRIEF

Mutations in *KRAS* and *BRAF* were mutually exclusive. Mutations in *KRAS* were otherwise associated with high age, MSS tumors and mutations in *PIK3CA* (exon 9). The V600E mutation in *BRAF* was associated with female gender, proximal tumor location, advanced stage and tumor grade, MSI and diploidy. Mutations in *PIK3CA* were associated with high age and proximal tumor location, and mutations in exon 20 were more common in MSI tumors.

None of the mutations had prognostic impact in the unstratified cohort. After clinically relevant stratification, a significant negative impact was identified for the V600E mutation in *BRAF* in colon cancer stage II, but only for MSS tumors. In females with colon cancer stage III, a negative prognostic effect of mutation in *KRAS* codon 13 was identified. Both findings have potential for clinical implication.

Conclusion:
The mutation status of the individual genes had no prognostic impact in the unstratified cohort, but identified clinically interesting subgroups of patients. The V600E mutation in *BRAF* was associated with inferior prognosis for patients with MSS colon cancer stage II, a subgroup which could benefit from adjuvant treatment. Mutations in *KRAS* codon 13 have negative prognostic impact in females with colon cancer stage III and these patients could be subjects for more aggressive treatment and follow-up.

DISCUSSION

Quality of the clinical and histopathological data

All clinical data in this thesis is extracted from the clinical database at Aker University Hospital. They have been collected prospectively by the attending physician in connection to discharge or follow-up by means of standardized forms. We believe this has increased the accuracy compared to a retrospective recovery of data.

Most doctors at the department have participated in the collection of clinical data, but just a few have been involved in registration and maintenance of the database, which has been done under supervision by Professor Arild Nesbakken for the whole period. In connection to different projects, the data of interest have been controlled against the hospital records as an extra control of accuracy. This has also been conducted in connection to this study, were we among other data, have controlled all collected information regarding lymph nodes against the original reports. The fact that the Hospital records have been available for repeated quality controls is a major advantage in this series. This has also made it possible to collect long term clinical data and update these regularly.

Both the clinical and pathological data are based on routine examinations and follow-up. A special program for patients included in research might result in more detailed and precise information, but would not be as representative for the everyday clinical setting.

Norway has central health registers of high quality, including the Norwegian Cancer registry and the Register of Cause of Death. Data from these have been used for control of inclusion and for collection of data missing from the hospital records.

Based on the arguments above, we believe that the clinical data included in this thesis is of very high quality. The collection of archive tissue is based on these clinical data and therefore represents a true consecutive, unselected series of tumor samples.

A weakness in the database is that some pathological markers with known or probable prognostic impact are not routinely registered. Tumor invasion in blood and lymphatic vessels have a proven negative effect on prognosis. For border configuration, lymphoid response to tumor, and perineural invasion, the documentation is not as strong, but they are promising markers for prognosis in colorectal cancer [157]. These morphological findings

have not been systematically documented in the pathology reports and are therefore not registered at a frequency that makes them useful in our analyses. Some of these deficiencies can be corrected by a re-examination of the histological sections from all tumors, but for some variables the standard sections now available might not be sufficient for a complete evaluation.

Formalin fixed paraffin embedded tissue - pro and cons

The analyzed tissue in this project is formalin fixed paraffin embedded (FFPE) archive material. Formalin has been known as a tissue fixative since 1893 and is an excellent preserver of morphology [276]. It does however induce cross links between nucleic acids and other tissue components which inevitably leads to fragmentation of DNA and RNA.

The protocols for formalin fixation at the department of Pathology at Aker University Hospital improved during the study period and buffered formalin was introduced. Here pH, salt, and formic acids are kept at physiological levels and this reduces the fragmentation of nucleic acids and stabilizes RNA and protein expression levels [276]. Further improvement of preservation of nucleic acids can be achieved by cold fixation, where the tissue is put in pre-cooled formalin and kept refrigerated at 4°C during fixation [276], but the department did not have the necessary facilities for this.

The average fragment length is 300-400 base pair for DNA and 200 bases for RNA after standard fixation and optimized extraction [277], and the best results of PCR-based RNA analyses are obtained for amplicon sizes less than 130 bp [278]. This is a major drawback of FFPE tissue compared to the higher quality of nucleic acids from fresh frozen tissue and makes it less suitable for genome wide analyses. Targeted DNA analyses based on polymerase chain reaction (PCR) are however widely used if the fragment of interest is within reasonable lengths, and FFPE tissue is therefore suitable for gene or mutation-specific studies. The nucleic acids, especially RNA, will slowly degrade after formalin fixation and the quantity and success rate of RNA extraction will deteriorate over time [279]. The extraction results can however be somewhat improved by increased incubation time with proteinase K [280].

The major advantage of FFPE tissue in cancer research is that it is readily available for all patients who have ever undergone biopsy or resection of their tumor. Pathology departments are obliged to keep the tissue for a minimum number of years before destruction, but in

practice such material is seldom destroyed. This means that tissue can be collected in retrospect and truly consecutive series can be obtained with complete long term clinical data.

Fresh frozen tissue is best suited for genome wide analyses. It is however rarely used in a routine setting and is usually collected only in relation to research projects. A written consent must therefore then be obtained before sampling and extra measures must be in place regarding tissue handling and storage. This is resource intensive and inclusion is seldom complete. It also makes it difficult to include patients in an emergency setting. To collect a large, high quality biobank series takes many years and even longer before long term clinical data are complete.

DNA extraction

For this large series of tissue samples, we needed a procedure that was robust, quick and easy to perform without compromising on the yield or quality of DNA and the DNA Mini kit from Qiagen was selected. This kit has comparable performance to other commonly used procedures [280], is easy to follow, and extraction is completed within 24 hours.

Marker panel for MSI determination

For analyses of microsatellite instability (MSI), mutation analyses of the five original Bethesda markers were performed [33]. The use of the three dinucleotide markers has been debated due to low sensitivity compared to the mononucleotide markers. A revised panel of markers including five mononucleotide markers has been suggested [35] and some report that the revised panel is more sensitive and easier to use than the original [281-283].

A multicenter study in 2010 assessed the interlaboratory reproducibility of the original and the revised panel as well as the accordance between the two panels [284]. It concluded that both panels gave a perfect agreement between laboratories, and that the concordance between the two panels was complete, given that MSI-L and MSS were grouped together. We therefore conclude that the original panel used in our analyses performs as well as the revised panel.

One potential weakness in our analyses is the lack of corresponding normal tissue. The used markers are not completely monomorphic and some variability according to ethnicity is known [285]. However our series include almost solely patients with a Scandinavian

background and expected variability was minimal. Four DNA samples from healthy Norwegian blood donors were therefore included as controls in each run.

The only available tissue that could have served as a normal in this series is the cancer-free tissue from the margin of the resected specimen. Given a possible field effect of some molecular changes and the risk of contamination during sampling, this would not be an ideal comparison and could lead to false negative results.

Mutation hot spots

Our aim was to look for clinically useful prognostic markers. This implied that the assays should be kept as simple as possible without losing important information about the analyzed genes. A few acknowledged hotspots for each gene was therefore chosen as targets based on the literature and the reported mutation frequency in the COSMIC database (http:// cancer.sanger.ac.uk/ cancergenome/projects/cosmic/).

Using the COSMIC database for confirmation of mutational hotspots is associated with some limitations. The number of mutations for each codon is based on reported data from different studies. The patients samples included in these studies might be biased by selection and not representative for the whole population. Until the recent development with deep sequencing, mutation analyses have rarely been done for the complete genes, but only for the parts where one expect to find mutations. Codons that are considered to be hotspots therefore tend to be analyzed more often than the rest of the gene and a reinforcement of possible hotspots must be expected. On the other hand, the COSMIC database represents the most comprehensive collection of identified mutations in cancer at the present.

Relevance of the study

To reduce mortality for those diagnosed with colorectal cancer, we have to improve early detection and treatment strategies. The latter might be achieved by better surgical techniques, improved radiation therapy or by introducing new and more effective drugs. But there is also a potential for improving the selection of patients who should be offered the adjuvant treatment already available today. Our study was initiated to pursue this matter.

The clinically most interesting group in colorectal cancer is stage II. The prognosis for patients in this group is good and Sjo et al. found a five year relative survival of 74% and 77% for men and women, respectively after resection of colon cancer with curative intent in our

series [150]. In studies of adjuvant treatment in stage II, there is a small, but non-significant improvement in survival [286]. If we could identify the patients with the highest risk of relapse within stage II, they could be offered the same adjuvant treatment as stage III and hopefully achieve improved survival.

In stage III, all fit patients up to 80 years receive adjuvant treatment although at least 50% are cured by surgery alone [150]. If it was possible to identify a group with superior prognosis within stage III, these could be spared the adverse effect of the treatment and the inconvenience of the regular visits to the hospital. On the other hand, if we could identify a group with inferior prognosis within stage III, even more aggressive treatment and follow-up could be indicated.

We wanted to explore if it was possible to utilize already available markers for subclassification within the TNM stages for more precise prognostication of patients. If so, a new treatment practice could be introduced accordingly and without unnecessary delay.

The prognostic impact of the selected markers was explored in the largest consecutive series of Norwegian colorectal cancer patients used for this purpose to our knowledge. With a prospective registration of comprehensive clinical data, long follow-up and archived FFPE tissue, this series was well suited for the purpose. It can always be argued that a larger series would have been even better. On the other hand, if you need several thousand patients to find an effect, the effect is either very small or affects only a small number of patients and therefore has less clinical relevance. We believe that the size of the current series is adequate for the purpose.

Our findings and their clinical impact

In paper I, **the number of examined lymph nodes was demonstrated to have significant impact on staging.** The identified association between the number of examined lymph nodes and the proportion of patients with stage III disease is in line with other studies [162, 163, 287]. Swanson et al. recommend that a minimum of 13 lymph nodes should be examined while Goldstein did not find such a cut point. A Working Party Report to the World Congress of Gastroenterology in 1991 recommended that a minimum of 12 lymph nodes should be examined before deeming a radical resection to be without lymph node metastasis [288].

DISCUSSION

The relationship between the number of examined lymph nodes and staging, explains the observation of more examined lymph nodes in patients with stage III disease; The more lymph nodes that are evaluated, the higher the probability of identifying at least one with metastasis [163].

In paper I we also documented that **the number of examined lymph nodes had prognostic impact in colon cancer stage II** and that survival was significantly different for patients with stage II and 0-7, 8-11, or ≥12 examined lymph nodes. Another Norwegian, but smaller study did not find that patients with less than 12 examined lymph nodes had inferior prognosis [289], but our finding is in line with several other studies [161-163] and a systematic review from 2007 [164]. This finding can be related to several issues. The number of examined lymph nodes can be regarded as a surrogate marker for the combined quality of the surgery and the pathological examination. Few nodes can be the result of inadequate surgery where metastatic nodes are left behind or incomplete pathological examination resulting in erroneous classification. In the first case, inferior prognosis can be due to residual cancer and for both cases, incorrect classification means that the patient is not offered adjuvant treatment as is standard treatment for colon cancer stage III. Erroneous classification will also affect the stage specific prognosis and a subsequent improvement of classification can lead to improved prognosis in line with the Will Rogers phenomenon (see box).

The Will Rogers phenomenon is obtained when moving an element from one set to another, raises the average values of both sets.

It is based on the following quote, attributed to American comedian William Penn Adair "Will" Rogers (1879–1935):

"When the Okies left Oklahoma and moved to California, they raised the average intelligence level in both states"

Based on the number of examined lymph nodes' impact on both staging and prognosis, it is fair to say that the high risk group within stage II includes all patients with less than 12 lymph nodes. The guidelines of the National Comprehensive Cancer Network (NCCN) also regard patients with colon cancer stage II and <12 examined lymph nodes as a high risk group and recommend adjuvant treatment for this group (http://www.nccn.org/professionals/physician_gls/f_guidelines.asp).

This stands in some contrast to the current Norwegian guidelines which recommend that a minimum of 12 lymph nodes should be removed and examined, but includes only patients with less than 8 examined lymph nodes in the high risk group that is offered adjuvant treatment. It should be considered if not all patients with <12 lymph nodes should be included in the high risk group and offered adjuvant treatment.

A third finding in paper I was that **the number of examined lymph nodes increased significantly in the study period**. Since D3 was the standard resection for colon cancer during the whole period, we believe that the observed improvement in our series is mostly due to improved routines at the pathology department. Regardless, this resulted in a non-significant **stage migration** in the same period, where the proportion of stage III increased at the cost of stage I and II. In updated information from the Norwegian Cancer Registry, a similar stage migration is described. In fact, during the last five decades, the proportion of stage III disease has increased from 28% in 1956-1960 to 55% in 2006-2010 [58]. This change has come gradually, but migration seems to be most evident in the last two decades.

This observed stage migration has implications for previous and current research. Older patient series are probably not comparable to current series because a proportion of the patients were understaged.

The observed variation in the number of examined lymph nodes within and among studies and over time can be related to several factors that can be categorized as patient specific, surgery related or connected to the histopathological examination [290].

In a large study from the British Islands, Tekkis et al evaluated clinical data from over 5 000 patients who underwent resection with curative intent from 2000 to 2002. They found that the number of examined lymph nodes were significantly associated with age, comorbidity (ASA grade), stage, type of resection, and preoperative radiotherapy in both uni- and multivariate analyses [291]. The association with age is also shown by others [290, 292-295]. In addition to less extensive surgery in the eldest due to comorbidity, Tekkis et al. introduced a theory of involution of nodes resulting in decreasing number and size of lymph nodes with increasing age to explain this finding [291].

In paper two, we found that **tumors located in the proximal colon were associated with a higher yield of lymph nodes** than tumors in the distal colon. Several other studies have demonstrated the same [289, 292, 294]. Variation in the proportions of proximal tumors

among series can therefore lead to different lymph node yield. Possible explanations for this finding include the anatomic distribution of lymph nodes and more extensive resections for tumors in the proximal colon. Tekkis et al. found that the standard and extended right hemicolectomy, typically performed for proximal colon tumors, had the highest lymph node yields and resections for sigmoid or distal rectal tumor had the lowest [291]. Shen et al. also measured the length of the resected specimen and found that when controlling for specimen length, proximal tumors had a higher number of lymph nodes than sigmoid and rectal cancers [293]. Based on these considerations, it should be evaluated if all types of resections should meet the same standard for examined nodes, as is custom today.

With the increasing focus on lymph node retrieval and examination, new guidelines for surgery and examination of the resected tissue have been established. In Norway, an increasing specialization in surgery has been conducted to increase the number of procedures for each surgeon, department and hospital to improve quality in all parts of the treatment [58]. For the pathologists, the demand for a minimum number of examined lymph nodes has resulted in a more standardized examination. The observed increase in the average number of examined lymph nodes in Norway and other countries over the last decades [163, 296] is most likely a result of this combined effort.

The main finding in paper II is that patients with **microsatellite instable (MSI) tumors have better prognosis** than those with stable tumors. This is in accordance with several studies including two systematic reviews and meta-analyses [128, 129]. The positive prognostic impact was however confined to stage II in our series while other series have diverging conclusions on this point. Halling et al found that the effect was confined to stage C (TNM stage III) in a population of Dukes stage B2 and C [262], Samowitz et al. found a positive prognostic impact of MSI in stage III and IV [297], while Lanza et al. found an impact for both stage II and III [298]. Most other studies do not find any prognostic impact of MSI when stratifying for stage or do not calculate and report it. This is can be due to small patient series where stratification inevitably leads to small groups without the necessary power to demonstrate statistically significant differences.

One possible interpretation of our finding is that the MSI phenotype are less prone to metastasize or at least needs more time to accumulate the necessary genetic and epigenetic changes for lymphatic and hematogenous spread compared to other phenotypes. This fits with our finding that the highest frequency of MSI is observed in stage II, a finding we share with other large series [228, 299, 300].

A strong immune response, observed as tumor infiltrating lymphocytes (TIL), has been suggested as an explanation for the good prognosis in patients with MSI-tumors [190, 301, 302]. MSI is caused by defect MMR which lead to insertions and deletions in repeat sequences. If the repeat sequence lies within the coding region of a gene, this results in a frame shift mutation. This leads to an abnormal coding sequence, changes in the amino acid sequence, and a premature termination codon (PTC). Most PTC-containing mRNAs are degraded by nonsense-mediated mRNA decay, unless the PTC is located in the last exon. Then the mRNA is translated, resulting in a truncated protein with a new peptid sequence [303]. These novel proteins, so called neopeptides, induce a specific T-cell response in vitro [304]. Another study found that the number of frame shift mutations were associated with the immune response in patient samples [305]. These observations account for the enhanced immune response observed in MSI tumors. If the enhanced immune response alone explains the superior prognosis in patients with MSI tumors remains to be settled.

Inhibition of epithelial mesenchymal transformation (EMT) due to mutations in transforming growth factor beta receptor 2 (TGFBR2) has also been proposed as an explanation of the superior prognosis in MSI tumors. EMT is involved in embryological development, tissue remodeling and wound repair, but is also regarded a necessity for migration, invasion and metastasis of tumor cells. The TGFBR2 gene contains an A_{10} repeat and has a mutation frequency of 75-80% in MSI tumors [304-307]. In early stages of carcinogenesis TGFBR2 plays the role as tumor suppressor [308], while it can induce EMT and thereby enhance tumor progression in later stages. This was demonstrated in a study from 2010 were Pino et al found that cells with mutation in TGFBR2 did not respond to TGF-B1, while cells with wild type TGFBR2 underwent EMT [309]. From this it can be expected that mutations in TFGBR2 will influence prognosis in MSI tumors, and several studies have been conducted to explore this [130, 306, 307, 310]. The results however show no effect of mutation, neither positive nor negative. If this is due to the dual effect of TGFBR2 or other circumstances is not known.

The positive prognostic impact of MSI is well documented by others, but we found that the prognostic effect was confined to stage II. It would be advantageous to validate this finding in a contemporary series. The immediate clinical relevance is, however, low since MSI identifies a subgroup with superior prognosis that is not included in routinely adjuvant treatment. Because of their good prognosis and lack of response to 5-FU [132], these patients should neither be included in clinical trials of adjuvant treatment with 5-FU monotherapy.

DISCUSSION

Most guidelines recommend adjuvant treatment to colon cancer stage II with high risk of recurrence, but the variables included in assessment of risk varies [311] (http://www.nccn.org/professionals/physician_gls/f_guidelines.asp#site), (http://www.helsebiblioteket.no/retningslinjer/kreft-i-tykktarm-og-endetarm/forord). In line with the Norwegian guidelines, I believe that patients with perforation close to tumor or with ≤8 examined lymph nodes should be included in adjuvant treatment due to increased risk of relapse, irrespective of tumor biology. Preferably, MSI-status should be assessed before treatment with 5FU monotherapy and oxaliplatin or irinotecan added if the tumor displays MSI.

If also patients with acute presentation due to obstruction or patients with positive resection margins (R1), tumor invasion into lymphatic or venous vessels, or less than 12 examined lymph nodes should be regarded as high risk patients and included in adjuvant therapy, is a subject for discussion.

Another finding in the second paper is that **the number of examined lymph nodes** is significantly associated with MSI, sex, age and stage in univariate analyses, but in multivariate analyses only age, tumor location and stage III have significant impact. The associations between lymph node yield and age, tumor location, and stage are discussed above. The association between MSI and the number of examined lymph nodes has also been reported by others [289, 312, 313] and the strong immune response in MSI tumors has been suggested as an explanation for this finding. With an active immune response, the local and regional lymph nodes can become larger and firmer and easier to identify in the mesocolic fat and this might ease the identification of lymph nodes for the pathologist. This can contribute to more accurate classification, stage migration and better stage-adjusted prognosis, as observed for MSI tumors. However, most studies examining the association between MSI and number of examined lymph nodes do not adjust for tumor location. In our study, we found that MSI did not have significant impact on the number of lymph nodes when adjusting for tumor location and other relevant factors. Future studies assessing the prognostic impact of MSI or the number of examined lymph nodes should adjust for tumor location to reduce the risk of bias.

Even though patients with MSI tumors have superior prognosis, some of these patients will also relapse and die of their colorectal cancer. It has been speculated whether this subgroup can be identified based on the specific genes that are mutated due to the mismatch repair

deficiency. So far, neither mutations in individual genes nor combinations of genes have demonstrated prognostic impact.

In paper III we explore the prognostic impact of mutations in *KRAS*, *BRAF*, *PIK3CA* and *PTEN*. Our main finding is that **the V600E mutation in *BRAF* has significant negative prognostic impact in stage II MSS colon cancer**. This effect is still significant when adjusting for sex, age, tumor grade, and urgency of surgery in a multivariate analysis. A negative prognostic impact of mutation in *BRAF* stratified for MSI status has earlier been demonstrated in some large series [228, 229, 314], but to our knowledge this is the largest consecutive series from one center to confirm this finding.

Mutations in *KRAS* and *BRAF* are mutually exclusive. Mutation in either of the genes leads to permanently active gene products and subsequent activation of the MAPK cascade. This is in turn involved in regulation of a wide range of cellular functions including transcription, differentiation and proliferation. It is therefore not surprising that these proto-oncogenes are frequently mutated in cancer. According to the COSMIC database, *KRAS* and *BRAF* are mutated in 22% and 19% of all reported tumor samples, respectively. *KRAS* are most frequently mutated in cancers of the pancreas (57%), large intestines (35%), and the biliary tract (26%), while *BRAF* mutations are most common in the thyroid (44%), skin (39%), and large intestines (12%) (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/ accessed August 29, 2013).

The patients identified by MSS and *BRAF* mutation represent a small but clinically very interesting group. In Norway, patients with colon cancer stage II are not offered any adjuvant treatment unless there is perforation of the gut or less than 8 examined lymph nodes. However, 25% will relapse and die within 5 years [150] and it is reasonable to assume that the combination of MSS and *BRAF* mutation identify some of these patients with inferior prognosis. Since MSS tumors are fully sensitive to 5-FU treatment, standard adjuvant treatment could improve the outcome for this group. This finding needs however confirmation in an independent consecutive series before these patients can be included in adjuvant treatment, and treatment should preferably be introduced in the form of a randomized clinical trial to document improved survival.

A negative prognostic impact of mutation in *KRAS* codon 13 in females with colon cancer stage III was also reported in paper III. This finding was confirmed in uni- and multivariate analyses of both OS and RFS in our series. Samowitz et al found negative impact of the most

common mutation in *KRAS* codon 13 (G13D) adjusting for age and stage, but the effect was not significant at a level of 95% confidence (p=0.08) [213]. For other large series exploring the prognostic impact of different mutations in *KRAS*, the results are negative or contradictory [144, 219, 315].

It should be noted that the number of patients are small and our finding needs verification in additional series, but if it is confirmed, a treatment change is warranted for this subgroup. They represent a small subgroup with poor prognosis and a more extensive follow-up can be indicated. The goal must be to reveal relapse as early as possible, hopefully at a stage when curative surgery is still possible. The most potent form of adjuvant treatment can also be indicated in older patients even if this is usually reserved for those younger than 70 years.

With increasing age, the renal function and hepatic microsomal oxidation rate are reduced, and there is a decrease in the distribution volume of hydrosoluble agents. These physiological changes lead to increased half-life of cytotoxic substances. Furthermore, the elderly have increased vulnerability of normal tissues like the hematopoietic system, the mucosa, the nervous system and the heart. In sum, the process of aging reduces the therapeutic margins and increases the risk for toxicity [316]. Because of this, older patients have been underrepresented in clinical trials, both for adjuvant treatment and for treatment in metastatic disease [317]. The data available for the oldest patients are therefore limited and might suffer from selection bias.

However, with increasing health and life expectancy in a population with a rising number of elderly, treatment strategies for patients >70-75 years become all the more important and several studies have evaluated the effect of adjuvant treatment in elderly compared to younger patients. Goldberg et al. found that patients ≥70 years had the same effect of FOLFOX4 as younger patients, and with the exception of a limited increase in neutropenia and thrombocytopenia, there were no difference in adverse events [318]. Jessup et al. found that of the 26 600 patients registered with stage III colon cancer in the National Cancer Data Base between 1990 and 2002, 5 898 were aged ≥80 years. The use of adjuvant treatment (5-FU and Levamisole/ Leucovorin) was lower among the oldest patients, but the effect on survival was equal to what was seen for younger patients [319]. Finally, Bouvier et al. assessed the impact of adjuvant treatment on quality of life in colorectal cancer patients ≥75 year. Using a French, population based cancer register, they found no difference in reported quality of life for those receiving adjuvant treatment compared to those who did not [320].

In Norway, the age limit for adjuvant treatment was 75 years up to 2005, but was then extended to include all fit patients up to 80 years. According to Statistics Norway, the calculated life expectancy for 80 year old women and men are 10 and 8 years, respectively (http://www.ssb.no/), and with increasing health among the oldest, it might be time to extend this limit even further. If relapse of colorectal cancer can be prevented without loss of quality of life, perhaps all fit patients should be considered for adjuvant treatment, regardless of age.

Given the central role in the RAS-RAF-MAPK pathway, it is almost surprising that not all activating mutations in *KRAS* have the same negative prognostic impact as we observe for mutations in *BRAF*. The ability of *KRAS* to activate the PI3K-AKT pathway in addition to RAF-MAPK enhances this imbalance. At the same time this finding is in a sense in concordance with the fact that mutations in *KRAS* have been identified at high frequency already in non-dysplastic aberrant crypt foci [107], implying that it is an early event in carcinogenesis, but not a driver for dysplasia.

Any attempt to try to explain this discrepancy between activating mutations in *BRAF* and *KRAS* will be speculations. However, based on the fact that mutations in *BRAF* and *KRAS* are mutually exclusive, a mutated *KRAS* must activate the MAPK cascade through a normal, non-mutated *BRAF*. This might introduce a limiting step in the effect of the mutation while the mutationally activated *BRAF* does not meet such obstacles. Mutated *BRAF* might consequently be a more effective activator of the MAPK pathway resulting in a more aggressive phenotype.

We did not find any prognostic impact of mutations in *PIK3CA*. This might be a correct result or a false negative due to small numbers (type II error). The prognostic impact of mutations in *PIK3CA* has previously only been evaluated in three series of more than 300 patients to our knowledge. Abubaker et al. screened 418 colorectal cancers for mutations in exon 9 and 20. They found aberrations in 51 (12%) of the samples and that mutations were correlated with stage and MSI-status, but not with overall survival [242]. In the study by Fariña Sarasqueta et al., hotspots in exon 9 and 20 of *PIK3CA* were successfully analyzed in a series of 616 colon cancer stage I-III. They found that mutations in exon 20 were associated with inferior prognosis in stage III, but no significant effect was seen in stage I and II [243]. Liao et al. successfully analyzed exon 9 and 20 in *PIK3CA* in 1 212 colorectal cancer samples from two large prospective cohorts. Seven patients (0.6%) with coexistent mutations in exon 9 and 20, demonstrated inferior prognosis in uni- and multivariate analyses, but the effect stratified by

stage was not possible to calculate due to small numbers [214]. Ogino et al. used the same patient cohorts as Liao when they identified *PIK3CA* mutations as a negative prognostic marker in a multivariate analysis, including colon cancer stage I-III with non-mutated *KRAS* [321]. With such diverging results among rather large series, it is fair to postulate that a prognostic impact of *PIK3CA* is either small or confined to very limited subgroups.

In paper III, frame shift mutations in *PTEN* were identified in only 12 (15%) of 78 MSI tumors and no prognostic value could be identified. Aberrations were identified in 7 (9%), 6 (8%) and 0 samples for the A_6 (exon 7), A_6, and A_5 (exon 8) repeats, respectively. Tougeron et al found that in a series of 61 MSI colorectal cancers, the A_6 repeats were mutated in 10% and 42% of samples, respectively. They also discovered that these mutations were associated with the density of tumor-infiltrating lymphocytes [305].

Based on the diverging results between our series and the one published by Tougeron et al., it is difficult to conclude on the role of these mutations in colorectal cancer. The number of MSI tumors is however limited in both series, and analyses in a larger series would give a better indication of the prognostic significance of *PTEN* mutations.

To identify target genes for frame shift mutations in MSI tumors, Duval et al. analyzed the mutation frequency of 25 equal mononucleotide repeats in cell lines and tumor samples. Repeats that were mutated in more than ≈12% of MSI tumor samples were classified as targets for mutation and presumed to play a role in MSI carcinogenesis. A lower mutation frequency was regarded as part of the elevated background instability [322]. This suggested cut-off might be used as a guide line in further research on the clinical significance of frame shift mutations in *PTEN*.

CONCLUSIONS

These studies have explored the prognostic impact of several readily available markers in a large consecutive series from one hospital with prospectively registered clinical data. These are our main conclusions regarding prognostic markers:

- The number of examined lymph nodes has prognostic impact in colon cancer stage II and III.
- Lymph node ratio has prognostic impact in colon cancer stage III.
- MSI has positive prognostic impact in colon cancer stage II.
- *BRAF* mutation has negative prognostic impact in colon cancer stage II, but only in MSS tumors.
- Mutation in *KRAS* codon 13 is a possible negative prognostic marker in colon cancer stage III for women.

Other observations of interest:

- An increase in the number of examined lymph nodes was observed in the study period resulting in a stage migration and contributing to improved prognosis.
- The number of examined lymph nodes is associated with tumor location, age and stage.
- The MSI phenotype is most common in tumors in the proximal colon and associated with female gender and poor differentiation.
- Mutations in *KRAS* are associated with MSS and the V600E mutation in *BRAF* is associated with MSI and diploidy.
- Mutations in *KRAS* and *BRAF* are mutually exclusive as are mutations in exon 9 and exon 20 in *PIK3CA*.

FUTURE PERSPECTIVES

Validation and use of prognostic markers

This thesis presents three prognostic molecular biomarkers in three different subgroups of colorectal cancer patients. We demonstrate a positive prognostic impact of MSI in sporadic colorectal cancer stage II. Since it is already known that MSI also is a negative predictor of effect of 5-FU monotherapy, our finding suggests that patients with stage II MSI tumors should not be included in future clinical trials of 5-FU-based adjuvant treatment.

The negative prognostic impact of *BRAF* mutations in MSS colon cancer has been shown in other studies, but identification of clinical subgroups where this finding has relevance, is usually missing. The present study demonstrates that the effect is confined to colon cancer stage II. This implies that the combination of the prognostic markers MSI and *BRAF* can be used to identify patients with inferior prognosis who according to the predictive value of MSI will have full effect of standard 5-FU-based adjuvant treatment. These patients should therefore be included in clinical trials to explore the benefit of this treatment.

A negative effect of mutations in *KRAS* codon 13 has been suggested in a few previous studies, but we find that the effect is restricted to women with colon cancer stage III and suggests that these patients should be considered for adjuvant treatment, regardless of age.

Within short, we intend to validate these findings in our own department, using an independent, contemporary patient series of fresh frozen tissue, collected at Oslo University Hospital since2005 (see below).

Further studies in this series

The current patient series of FFPE tumor tissue is well suited as a validation series for different molecular markers. Specific mutations and epigenetic changes can be analyzed in the extracted DNA and the relation to clinical and histopathological variables assessed, as well as the impact on clinical endpoints. Due to the size of the series and the range of clinical data, the impact can also be calculated for relevant subgroups and adjusted for other variables.

Ploidy, assessed by image cytometry, has already been analyzed and a manuscript from that study is included as an appendix to this thesis.

A tissue micro array (TMA) has also been constructed from this series. This makes it possible to perform large scale expression analyses of protein and micro RNA (miRNA), both as separate projects, and to complement the data from genetic and epigenetic analyses.

The registration of morphological and pathological variables in this series is good, but not optimal. If the available histological sections are adequate, a re-examination to ensure a systematic and complete registration of lymphovascular invasion, perineural invasion, tumor border configuration, and host lymphoid reaction, would be very valuable, also in future comparison with molecular data.

Contemporary patient series

There is a constant need for contemporary patient series in the research on diagnostic, prognostic and predictive markers, both clinical and biological. As demonstrated in paper I, classification and prognosis change over time and older series are therefore not always relevant for current research and treatment. To prospectively collect and register clinical and pathological data along with fresh tissue is resource-demanding, but necessary for high quality research and should maybe be a part of the daily routine in all (University) hospitals.

From 2005 our team has collected a consecutive series of fresh frozen tissue from patients who undergo elective surgery for colorectal cancer at Oslo University Hospital. The objective is 1000 patients and so far samples from approximately 500 patients has been included along with comprehensive clinical data.

Series of FFPE tissue should also be collected regularly for validation of markers in series with long follow-up, and even if fresh tissue is most appropriate for modern high throughput analyses, FFPE can also be used in this setting. The patients included in molecular analyses in this thesis, underwent surgery between 1993 and 2003. Later, all patients up to 2012 have been identified and tissue samples retrieved from the archives.

Ongoing and future projects

This thesis is part of a research program for prognostic and predictive biomarkers for colorectal cancer. This involves working in a multidisciplinary team, including surgeons,

oncologists, pathologists, and biologists with different areas of expertise, to design and perform robust and clinically relevant projects. Analyses are performed utilizing a wide range of technology, of which most are available in own lab. Large-scale -omics analyses produce huge amounts of data, and these are processed and interpreted by team members skilled in bioinformatics.

The goal is to produce high quality translational research resulting in identification of prognostic and predictive biomarkers, or panels of markers, for clinically relevant subgroups of colorectal cancer patients. With our position within a comprehensive cancer center and the close collaboration with clinicians, we hope to shorten the time from discoveries in the lab to implementation of biomarkers in the clinic.

A study has also been initiated to increase our understanding of the clonal development and the scope of intratumoral heterogeneity in colorectal cancer. Repeated sampling from the patients included in this study gives a longitudinal series that will be analyzed in this work. Comparing results from different points of disease progression in the same patient will give new insight in the chronological development of colorectal cancer and potentially identify new biomarkers and targets for therapy.

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PAPER I

Prognostic impact of lymph node harvest and lymph node ratio in patients with colon cancer

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PAPER II

Microsatellite instability has a positive prognostic impact on stage II colorectal cancer after complete resection: results from a large, consecutive Norwegian series

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Marianne A. Merok. Terje Ahlquist. Ellen C. Royrvik, Kjersti F. Tufteland, Merete Hektoen, Ole H. Sjo, Tom Mala, Aud Svindland, Ragnhild A. Lothe, Arild Nesbakken

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Microsatellite instability has a positive prognostic impact on stage II colorectal cancer after complete resection: results from a large, consecutive Norwegian series

M. A. Merok[1,2,3], T. Ahlquist[2,3], E. C. Røyrvik[2,3], K. F. Tufteland[4], M. Hektoen[2,3], O. H. Sjo[1], T. Mala[1], A. Svindland[4], R. A. Lothe[2,3] & A. Nesbakken[1,3]

[1]Department of Gastrointestinal Surgery, Oslo University Hospital—Aker, Oslo; [2]Department of Cancer Prevention, Oslo University Hospital, The Norwegian Radium Hospital, Oslo; [3]Center for Cancer Biomedicine, Faculty of Medicine, University of Oslo; [4]Department of Pathology, Oslo University Hospital, Oslo, Norway

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Background: Microsatellite instability (MSI) was suggested as a marker for good prognosis in colorectal cancer in 1993 and a systematic review from 2005 and a meta-analysis from 2010 support the initial observation. We here assess the prognostic impact and prevalence of MSI in different stages in a consecutive, population-based series from a single hospital in Oslo, Norway.

Patients and methods: Of 1274 patients, 952 underwent major resection of which 805 were included in analyses of MSI prevalence and 613 with complete resection in analyses of outcome. Formalin-fixed tumor tissue was used for PCR-based MSI analyses.

Results: The overall prevalence of MSI was 14%, highest in females (19%) and in proximal colon cancer (29%). Five-year relapse-free survival (5-year RFS) was 67% and 55% ($P = 0.030$) in patients with MSI and MSS tumors, respectively, with the hazard ratio (HR) equal to 1.60 ($P = 0.045$) in multivariate analysis. The improved outcome was confined to stage II patients who had 5-year RFS of 74% and 56% respectively ($P = 0.010$), HR = 2.02 ($P = 0.040$). Examination of 12 or more lymph nodes was significantly associated with proximal tumor location ($P < 0.001$).

Conclusions: MSI has an independent positive prognostic impact on stage II colorectal cancer patients after complete resection.

Key words: adenocarcinoma, colorectal neoplasms, lymph nodes, microsatellite instability, prevalence, prognosis

introduction

Colorectal cancer is among the most common malignancies in the western world [1] and is becoming more common in developing countries as they approach a western lifestyle [2]. In Norway, the age-adjusted incidence rate has doubled over the last 50 years and is now among the highest in Europe [3].

Several clinical and pathological factors have prognostic impact on colorectal cancer including tumor stage, residual tumor (R-) status [4], tumor differentiation [5, 6], bowel perforation and emergency surgery [7]. In colon cancer, the number of examined lymph nodes has a prognostic impact [8–11]. Risk stratification according to these clinicopathological factors is applied to select patients for (neo-) adjuvant treatment. In Norway, stage III colon cancer patients with age less than 76 years are offered adjuvant chemotherapy. Stage II patients do not receive such therapy, except those with bowel perforation or less than nine examined lymph nodes after a thorough examination of the resected tissue. In rectal cancer, preoperative radiochemotherapy is recommended if the distance from the tumor or a metastatic lymph node to the mesorectal fascia is ≤3 mm, evaluated by magnetic resonance imaging.

However, current risk stratification does not adequately identify patients with good and poor prognosis. The 5-year relative survival rate of stage III colon cancer patients was 57% before adjuvant chemotherapy became standard treatment [3], which implies that more than half of these patients are cured by surgery alone and are over-treated when given adjuvant therapy. Five-year relative survival in stage II colon cancer is 75% [12], indicating that 25% of the patients relapse and die of cancer within 5 years after surgery. Possibly, adjuvant therapy for high-risk stage II patients might improve these results. Several biomarkers have been proposed to improve the identification of patients at risk of relapse, but none are implemented in clinical practice [13].

*Correspondence to: Prof. A. Nesbakken, Department of Gastrointestinal Surgery, Oslo University Hospital, P. O. Box 4950 Nydalen, N-0424 Oslo, Norway. Tel: +47-911-41-398; Fax: + 47-230-15-260; E-mail: arild.nesbakken@medisin.uio.no

Approximately 15% of all colorectal cancers display microsatellite instability (MSI), a molecular phenotype caused by defect mismatch repair [14–17]. In Lynch syndrome (former HNPCC), MSI is due to germline mutation in one of the MMR genes, usually *MLH1* or *MSH2* [18–20]. In sporadic colorectal cancer, MSI is mainly caused by epigenetic silencing of *MLH1* [21–23] and is characterized by poor differentiation, tumor-infiltrating lymphocytes, location in the proximal colon and association with female gender and age [14, 16, 17, 24–28].

We initially reported MSI as a marker of good prognosis in 1993 [14]. Subsequent reports have shown conflicting results; however, a systematic review from 2005 concluded that patients with MSI tumors have better prognosis than those with MSS tumors [29] and a meta-analysis from 2010 confirmed this finding [30]. It is yet to be decided whether this is valid for all stages, and the results from different studies differ at this point [24, 25, 28]. The aim of the present study was to evaluate the prognostic impact of MSI adjusted for stage and other clinical variables in a large, consecutive series from a single hospital.

materials and methods

Oslo University Hospital, Aker has a defined catchment area of 270 000 inhabitants. All patients with colorectal cancer admitted to the hospital in the period 1993–2003 were registered and clinical data recorded in a database. Registration has been controlled against the Norwegian Cancer Registry.

Major resection was defined as removal of the tumor-bearing bowel segment with the lymphovascular pedicle and mesentery with regional lymph nodes. Total mesorectal excision was carried out in all patients with rectal cancer. Fifteen percent of the patients underwent emergency surgery, due to obstruction or perforation of the bowel.

TNM-staging followed the UICC/AJCC system, version 5, for all patients. Based on the radiological examinations, intraoperative findings and macroscopic and microscopic examination, the resection was classified as R0 (complete resection/no residual tumor), R1 (microscopic residual cancer at the resection margin) or R2 (macroscopic or radiological evidence of residual cancer, locally or distant). For colon cancer, the total number of examined lymph nodes was registered.

The patients were split into three subgroups according to tumor location: proximal colon including the cecum through the transverse colon; distal colon including the left flexure through the rectosigmoid flexure; rectum was defined as the bowel up to 15 cm above the anal verge.

Colon cancer patients with age less than 76 years and all rectal cancer patients who underwent curative surgery entered a 5-year follow-up program (supplementary Table S1, available at *Annals of Oncology* online). Patients who were not enrolled in systematic follow-up would be admitted to our hospital if developing symptoms of relapse, implying that most relapses would be identified and registered. Information about death was retrieved from the Norwegian Tax Administration.

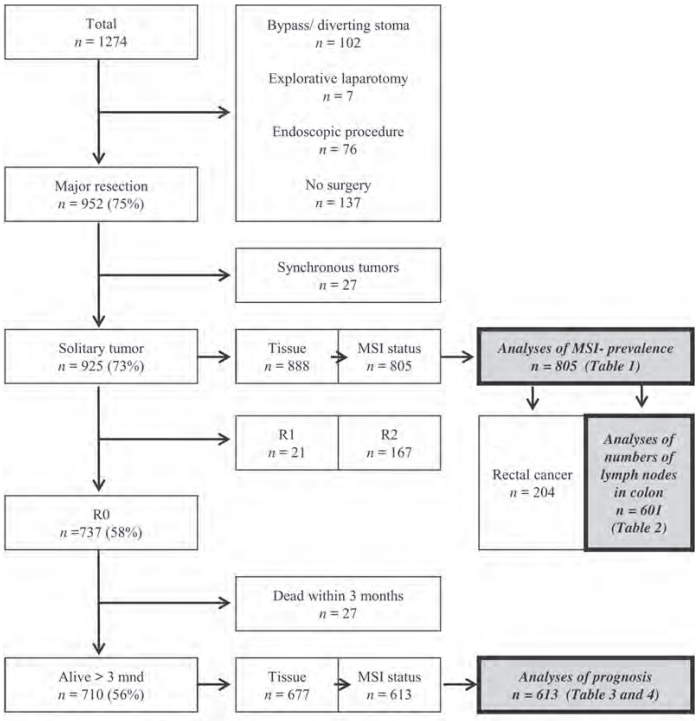


Figure 1. Flow chart for all patients with colorectal cancer admitted to Oslo University Hospital, Aker, in the period 1993–2003.

Formalin-fixed paraffin-embedded tumor tissue was retrieved for all patients who had undergone major resection, and HE sections were re-examined to confirm the presence of cancer and mark the most representative area. Four 25 μm sections were used for DNA extraction with QIAamp DNA Mini kit from Qiagen (GmbH, Hilden, Germany). The method was modified by adding an early step for removal of paraffin by heating to 90°C for 10 min after buffer was added.

For determination of the MSI status, microsatellite analyses were carried out for the five loci recommended by the National Cancer Institute [31]. PCR for the mononucleotides (BAT25 and BAT26) and the dinucleotides (D2S123, D5S346 and D17S250) were run separately. Both the reactions used 37 ng DNA templates in a 10 μl reaction volume consisting of a 1× Multiplex PCR Master mix (buffer, 1.5 mM $MgCl_2$, nucleotides and enzyme, QUIAGEN GmbH, Hilden, Germany), fluorescent primers and water. The mononucleotide markers underwent 30 cycles and the dinucleotide markers 35 cycles. Fragment analysis was accomplished on 3730 Genetic Analyzer (Applied Biosystems, Life Technologies, Carlsbad, California). Four DNA samples extracted from blood of healthy donors were included in each run as controls. The results were scored independently by two observers. The MSI status for each locus was determined after two independent runs with the same conclusion (MSI or wild type). If there were contradictory results, the locus was scored as 'not determined'. Samples with two or more loci exhibiting abnormal allelic ranges were scored as MSI high (MSI-H, from here on referred to as MSI). If one locus was MSI and four loci were wild type, the sample was scored as MSI low (MSI-L). Samples with wild type in all five loci were scored as microsatellite stable (MSS). For further analyses, MSI-L and MSS were included in the same group, and referred to as MSS, as were samples with four wild-type loci and one 'not determined' locus.

The associations between MSI, number of examined lymph nodes and different clinical variables were explored in contingency tables, and Pearson's chi-square test was applied. Logistic regression was used in multivariate models to explore different variables' impact on the MSI-status and the number of examined lymph nodes.

The prognostic impact of MSI and clinical variables was analyzed with 5-year overall survival (5-year OS) as primary endpoint; death from any cause was defined as event and patients were censored 5 years after surgery. The second endpoint was 5-year relapse-free survival (5-year RFS); deaths from any cause and recurrence (locally and/or distant) were defined as events [32]. The patients were censored at loss to follow-up, defined as the last date for clinical or radiological examination or at 5 years after surgery. Survival analyses were carried out using the Kaplan–Meier method, and the survival distributions were compared with the log-rank test. Multivariate analyses were carried out using Cox regression analyses, all

Table 1. Prevalence of MSI according to clinical and histopathological variables ($n = 805$)

		Univariate[a]		Multivariate[b]		
Variables	Total *N* (%)	MSI *N* (%)	*P*	OR	95% CI	*P*
Total	805	112 (14)				
Sex						
Female	431 (54)	82 (19)	<0.001	Ref		
Male	374 (46)	30 (8)		0.41	0.24–0.70	0.001
Age						
<60 years	146 (18)	18 (12)	0.241	Ref		
60–70 years	164 (20)	16 (10)		0.42	0.18–1.00	0.051
70–80 years	300 (37)	46 (15)		0.61	0.30–1.24	0.174
>80 years	195 (24)	32 (16)		0.56	0.26–1.19	0.131
Tumor location						
Proximal colon	327 (41)	96 (29)	<0.001	Ref		
Distal colon	274 (34)	12 (4)		0.14	0.07–0.27	<0.001
Rectum	204 (25)	4 (2)		0.05	0.02–0.13	<0.001
Stage						
I	118 (15)	7 (6)	<0.001	Ref		
II	323 (40)	65 (20)		1.89	0.75–4.75	0.176
III	210 (26)	27 (13)		1.07	0.40–2.88	0.887
IV	154 (19)	13 (8)		0.83	0.17–4.03	0.818
Histopathologic grade						
G1 + G2	685 (85)	65 (10)	<0.001	Ref		
G3	102 (13)	42 (41)		7.34	4.06–13.27	<0.001
Mucinous	9 (1)	4 (44)		4.93	1.12–21.71	0.035
Surgery						
Elective	683 (85)	101 (15)	0.090	Ref		
Acute	122 (15)	11 (9)		0.44	0.21–0.95	0.038
Residual tumor						
R0	637 (79)	97 (15)	0.061	Ref		
R1	17 (2)	3 (18)		1.21	0.24–6.10	0.813
R2	151 (19)	12 (8)		0.37	0.10–1.46	0.157

[a]Contingency tables, chi-square test.
[b]Logistic regression, all included variables are displayed in the table.

variables from univariate analyses were entered into the models. A *P*-value of <0.05 was considered statistically significant. All analyses were carried out with SPSS 16.0 (IBM®SPSS®, IBM Corporation, Armonk, New York).

The study was carried out according to the Helsinki declaration and approved by the Regional Ethics Committee for Medical Research (REK approval 1.2005.1629) and the Norwegian Data Inspectorate.

results

The selection of patients included in the study is illustrated in Figure 1 and the characteristics of the cohorts included in the different analyses are displayed in the supplementary Table S2, available at *Annals of Oncology* online. A total of 1274 patients were admitted with colorectal cancer from 1993 to 2003 and 925 patients underwent major resection of a solitary tumor. Tumor tissue was available from 888 and the MSI status was successfully determined in 805 (91%) patients who were included in the analyses of MSI prevalence.

MSI prevalence and clinical variables

MSI was demonstrated in 112 (14%) patients (Table 1). MSI tumors were most frequent in the proximal colon and 86% of the MSI tumors were located proximal to the splenic flexure. MSI was more common in females who had a greater proportion of their tumors in the proximal colon (49% versus 31% in men, $P < 0.001$), but also had a higher frequency of MSI in their proximal tumors (34% versus. 20% in men, $P = 0.005$). The prevalence of MSI varied with tumor stage with the lowest frequency in stage I (6%) and the highest in stage II (20%). This was partly because stage I tumors were rare in the proximal colon ($n = 25$, 8%), whereas stage II tumors were frequent ($n = 145$, 44%). Including only proximal colon cancers, the frequencies of MSI in stage I ($n = 25$), stage II ($n = 145$), stage III ($n = 82$) and stage IV ($n = 75$) were 24%, 39%, 26% and 16%, respectively. MSI was most prevalent in tumors with poor differentiation (G3) and in mucinous tumors. In a multivariate analysis (Table 1), MSI was associated with female gender, tumor location in proximal colon, poor differentiation and elective surgery.

MSI and number of examined lymph nodes

In the analyses of number of lymph nodes, rectal cancer patients were excluded, leaving 601 colon cancer patients. Because of missing data for three patients, 598 patients were included in the analyses. Twelve or more examined lymph nodes were obtained in 31% of the patients and the

Table 2. Proportion of colon cancer patients with ≥12 examined lymph nodes (ln) according to clinical and histopathological variables ($n = 598$)

Variables	Total	Univariate[a]		Multivariate[b]		
	N (%)	≥12 ln *N* (%)	*P*	OR	95% CI	*P*
Total	598	186 (31)				
MSI status						
MSI	108 (18)	46 (43)	0.004	Ref		
MSS	490 (82)	140 (29)		0.86	0.54–1.37	0.534
Sex						
Female	337 (56)	117 (35)	0.030	Ref		
Male	261 (44)	69 (27)		0.73	0.50–1.07	0.105
Age						
<60 years	92 (15)	41 (45)	0.019	Ref		
60–70 years	114 (19)	33 (29)		0.53	0.30–0.93	0.027
70–80 years	224 (38)	60 (27)		0.43	0.26–0.69	<0.001
>80 years	168 (28)	52 (31)		0.48	0.29–0.80	0.005
Tumor location						
Proximal colon	324 (54)	128 (40)	<0.001	Ref		
Distal colon	274 (46)	58 (21)		0.45	0.30–0.67	<0.001
Stage						
I	64 (11)	14 (22)	0.004	Ref		
II	249 (42)	78 (31)		1.60	0.95–2.68	0.075
III	153 (26)	63 (41)		2.50	1.44–4.35	0.001
IV	132 (22)	31 (24)		1.04	0.56–1.92	0.906
Histopathologic grade						
G1 + G2	498 (85)	155 (31)	0.903	Ref		
G3	83 (14)	27 (33)		0.87	0.50–1.50	0.611
Mucinous	8 (1)	3 (38)		1.11	0.25–4.86	0.888
Surgery						
Elective	482 (81)	153 (32)	0.284	Ref		
Acute	116 (19)	33 (28)		0.90	0.56–1.46	0.674

[a]Contingency tables, chi-square test.
[b]Logistic regression, all included variables are displayed in the table

distribution according to clinical variables is presented in Table 2. When including only tumors from the proximal colon ($n = 324$), the numbers of patients with 12 or more examined lymph nodes were 43 (45%) and 85 (37%) for MSI and MSS, respectively ($P = 0.203$). If only including MSS tumors ($n = 490$), the numbers with 12 or more lymph nodes were 85 (37%) and 55 (21%) for proximal and distal colon, respectively ($P < 0.001$). In multivariate analyses, age, tumor location and stage had a significant impact on the proportion with 12 or more examined lymph nodes, whereas the MSI status had no significant impact.

MSI and survival

The MSI status was successfully determined in 613 patients with solitary tumors who survived for >3 months after an R0-resection (Figure 1). These were included in the prognostic analyses, and matched well with all patients who underwent major resection with regard to age, gender and tumor location (supplementary Table S2, available at *Annals of Oncology* online). The group included 17 stage IV patients who underwent R0-resection of synchronous, distant metastases during or shortly after the primary operation.

Of the 613 patients included in the prognostic analyses, 157 (26%) experienced relapse and 224 (37%) died without known relapse. The 5-year estimated relapse rates were 10%, 23% and 42% in stages I–III, respectively according to the Kaplan–Meier method. For patients who survived without relapse, the median follow-up time was 65 months.

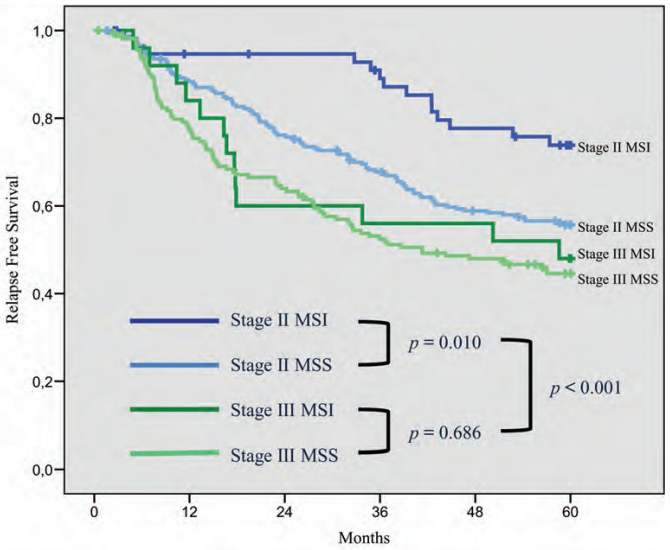
The 5-year OS rates were 69% and 61% for patients with MSI tumors and MSS tumors, respectively ($P = 0.214$), with the hazard ratio (HR) equal to 1.47 ($P = 0.112$). However, MSI was associated with significantly improved 5-year RFS (Table 3). Subgroup analyses demonstrated that the improved outcome for MSI tumors only applied to stage II, whereas no difference in the outcome was found in stage III (Figure 2). For stage I and IV, the numbers of MSI tumors were too small to draw any conclusions.

The prognostic impact of MSI status in stage II patients is presented in Table 4, showing 5-year RFS of 74% and 56% ($P = 0.01$) in MSI and MSS patients, respectively, with the HR equal to 2.02 ($P = 0.040$).

Table 3. Five-year relapse-free survival (5-year RFS) in stage I–IV colorectal cancer (R0-resection, solitary tumor, alive >3 months after surgery, $n = 613$)

		Univariate[a]		Multivariate[b]		
Variables	Total *N* (%)	5-year RFS (%)	*P*	HR	95% CI	*P*
Total	613	56.5				
MSI status						
MSI	92 (15)	67.1	0.030	Ref		
MSS	521 (85)	54.7		1.60	1.01–2.52	0.045
Sex						
Female	321 (52)	58.3	0.488	Ref		
Male	292 (48)	54.6		1.10	0.85–1.43	0.451
Age						
<60	111 (18)	74.8	<0.001	Ref		
60–70	126 (21)	60.7		1.88	1.17–3.04	0.010
70–80	236 (39)	53.4		2.40	1.56–3.70	<0.001
>80	140 (23)	43.4		2.92	1.83–4.67	<0.001
Tumor location						
Proximal colon	238 (39)	59.5	0.284	Ref		
Distal colon	198 (32)	53.3		1.24	0.91–1.71	0.179
Rectum	177 (29)	56.1		1.51	1.07–2.13	0.019
Stage						
I	117 (19)	75.0	<0.001	Ref		
II	291 (48)	59.2		1.95	1.27–3.01	0.002
III	188 (31)	45.0		3.37	2.18–5.21	<0.001
IV	17 (3)	11.8		5.55	2.88–10.70	<0.001
Histopathologic grade						
G1/G2	534 (87)	58.2	0.025	Ref		
G3	66 (11)	45.1		1.84	1.24–2.73	0.003
Mucinous	7 (1)	57.1		1.31	0.42–4.15	0.642
Surgery						
Elective	544 (89)	58.2	0.004	Ref		
Acute	69 (11)	43.1		1.35	0.94–1.96	0.107

[a]Kaplan–Meier estimate, log-rank test.
[b]Cox Regression, all included variables are displayed in the table.



Time (months)		12	24	36	48	60
Stage II						
MSI (58)	Events	3	3	6	12	14
	At risk	52	51	47	41	35
MSS (233)	Events	27	55	74	94	101
	At risk	203	175	153	101	118
Stage III						
MSI (25)	Events	4	10	11	11	13
	At risk	21	15	14	14	11
MSS (163)	Events	36	57	75	82	87
	At risk	122	101	82	74	63

Figure 2. Five year relapse-free survival (RFS), stage II and III, $n = 479$.

discussion

The important finding in the present study was that stage II patients with MSI tumors have better outcome than patients with MSS tumors. This is in accordance with several other publications [24, 28–30, 33–35]. This was demonstrated in a large, consecutive and population-based series with minimal risk of selection bias. The comprehensive set of clinical data made it possible to adjust for several well-known prognostic factors. Patients with synchronous tumors were excluded because of the uncertainty regarding which tumor was most relevant for prognosis. We chose robust endpoints according to Punt et al. [32] and end points based on the cause of death were not considered due to the risk of bias due to erroneous cause of death. Analyses were restricted to 5-year survival, as most deaths after this time will not be cancer related. Patients were censored at the time of the last examination with regard to recurrence, and bias due to loss of follow-up was minimized. This report follows the recommendations for tumor marker prognostic studies [36]. Based on these conditions, the conclusion with regard to the prognostic impact of MSI is reliable.

The positive prognostic impact of MSI was confined to stage II patients. In contrast, Samowitz et al. found significant impact only in stage III patients in a study of 1000 colon cancer patients from California and Utah, all less than 79 years of age, and with different ethnic background [28]. Benatti et al. presented a series of 1263 colorectal cancer patients and found a positive prognostic impact of MSI in stage II and III [24]. Patients with clinical suspicion of hereditary colorectal cancer syndromes were also included in this study and the mean age was only 65 years. The prevalence of MSI was unusually high (20%). The current series has the advantage of not being biased by any selection among the enrolled patients.

From 1997, patients up to 75 years with stage III colon cancer receive 5FU-based adjuvant treatment. A systematic review with meta-analysis from 2009 reported that MSI tumors do not respond to this treatment [37] and this could

Table 4. Five-year relapse-free survival (5-year RFS) in stage II colorectal cancer (R0-resection, solitary tumor, alive > 3 months after surgery, $n = 291$)

Variables	Total	Univariate[a]		Multivariate[b]		
	N (%)	5-year RFS (%)	*P*	HR	95% CI	*P*
Total	291	59.2				
MSI status						
MSI	58 (20)	73.8	0.010	Ref		
MSS	233 (80)	55.7		2.02	1.03–3.95	0.040
Sex						
Female	156 (54)	60.5	0.677	Ref		
Male	135 (46)	57.7		1.06	0.72–1.56	0.782
Age						
<60	46 (16)	79.9	<0.004	Ref		
60–70	53 (18)	65.4		1.91	0.84–4.32	0.122
70–80	118 (41)	53.9		2.91	1.42–5.97	0.004
>80	74 (25)	50.3		3.15	1.48–6.73	0.003
Tumor location						
Proximal colon	133 (46)	64.9	0.010	Ref		
Distal colon	91 (31)	58.1		1.18	0.73–1.91	0.505
Rectum	67 (23)	49.5		2.23	1.33–3.74	0.002
pT stage						
3	272 (93)	59.6	0.458	Ref		
4	19 (7)	52.6		1.72	0.84–3.50	0.138
Histopathologic grade						
G1/G2	250 (86)	59.3	0.756	Ref		
G3	32 (11)	62.8		1.61	0.79–3.30	0.190
Mucinous	6 (2)	66.7		1.41	0.32–6.17	0.647
Surgery						
Elective	252 (87)	61.2	0.018	Ref		
Acute	39 (13)	45.7		1.81	1.07–3.08	0.028

[a]Kaplan–Meier estimate, log-rank test.
[b]Cox regression, all included variables are displayed in the table.

camouflage an otherwise better prognosis for MSI tumors in stage III in our series. The patients who have received adjuvant treatment comprise 56 patients of whom 11 had MSI tumors. Excluding these from the analyses did not result in increased prognostic impact of MSI in stage III (data not shown).

The clinical applicability of MSI as a prognostic marker remains to be decided. Clearly, stage II tumors in the proximal colon make up the interesting subgroup because of the high prevalence of MSI (38%). Stage II patients do not routinely receive adjuvant therapy according to Norwegian guidelines. This seems reasonable for patients with an expected 5-year relative survival of 75% [12]. However, the MSS subgroup of patients had significantly worse prognosis, and these patients might benefit from adjuvant therapy. To demonstrate such a benefit, a randomized trial is necessary. Additional molecular markers may refine the poor and good MSI-based prognostic groups such as the recent ColoGuideEx, a 13 gene expression signature specific to stage II patients published by our group [38].

The prevalence of MSI in the current series was 14%. This is in accordance with comparable series [33, 39–42]. The previous documented association of MSI phenotype with right-sided colorectal cancer was confirmed. MSI was also more common in women than in men, partly due to the fact that women had a higher proportion of their tumors in the proximal colon (49%) compared with men (31%), which is in agreement with a study from New Zealand [43], but also because women had a higher frequency of MSI in their proximal tumors than men.

We found no significant association between MSI status and age. Other studies report the highest frequencies of MSI tumors in the oldest patients [28, 33, 44].

The proportion of MSI tumors was highest in stage II. This observation is in compliance with several other studies [24–26, 28, 40, 42]. The low number of MSI tumors in stage I in the present series can partly be explained by few stage I tumors in the proximal colon and numerous stage I tumors in the rectum. This finding might be connected to the absence of systematic screening for colorectal cancer in Norway which implies that most patients have developed symptoms at the time of diagnosis. Tumors in the proximal colon typically cause more subtle symptoms than tumors in the distal colon and rectum and may have reached a more advanced stage by the time of detection. The high frequency of MSI in stage II tumors might also reflect a less aggressive phenotype with lower tendency to metastasize [25].

The number of examined lymph nodes was low in this series, but probably representative for consecutive series from a

routine setting in this period. However, the low number should not introduce any bias in the calculations since this influences MSI/MSS and different tumor locations equally. Other authors have reported a higher number of examined lymph nodes in MSI patients [45–47], and suggested that MSI tumors induce larger lymph nodes which are more easily identified and retrieved by the pathologist. However, when adjusting for tumor location, the effect of MSI disappeared [47]. This is in line with our finding. A probable explanation is that different tumor locations result in different anatomical resections with unequal numbers of lymph nodes due to the anatomical distribution of mesocolic lymph nodes.

There is a correlation between the number of examined lymph nodes and correct staging [9], and this might explain why stage III patients have the highest number of examined lymph nodes. The correlation between the number of examined lymph nodes and age has also been described by others [10]. In the present series, a higher proportion of patients <60 years in the more recent years, corresponding to a period with increasing number of examined lymph nodes [48], might explain this.

In conclusion, the present study demonstrates that MSI is a positive prognostic factor in patients with stage II colon cancer, but not in stage III. MSS could be a clinical useful biomarker for the identification of patients with stage II right-sided colon cancer at increased risk of relapse.

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disclosure

The authors have declared no conflicts of interest.

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VEGFR-2, CXCR-2 and PAR-1 germline polymorphisms as predictors of survival in pancreatic carcinoma

F. G. Uzunoglu[1,†], J. Kolbe[1,†], H. Wikman[2], C. Güngör[1], B. A. Bohn[1], M. F. Nentwich[1], M. Reeh[1], A. M. König[1], M. Bockhorn[1], A. Kutup[1], O. Mann[1], J. R. Izbicki[1] & Y. K. Vashist[1*]

Departments of [1]General, Visceral and Thoracic Surgery; [2]Tumour Biology, University Medical Center of Hamburg-Eppendorf, Hamburg, Germany

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Background: Hypoxic environment of pancreatic cancer (PC) implicates high vascular in-growth, which may be influenced by angiogenesis-related germline polymorphisms. Our purpose was to evaluate polymorphisms of vascular endothelial growth factor receptor 2 (VEGFR-2), CXC chemokine receptor 2 (CXCR-2), proteinase-activated receptor 1 (PAR-1) and endostatin (ES) as prognostic markers for disease-free (DFS) and overall survival (OS) in PC.
Patients and methods: Genotyping of 173 patients, surgically treated for PC between 2004 and 2011, was carried out by TaqMan® genotyping assays or polymerase chain reaction. Chi-square test, Kaplan–Meier estimator and Cox regression hazard model were used to assess the prognostic value of selected polymorphisms.
Results: VEGFR-2 −906 T/T and PAR-1 −506 Del/Del genotypes predicted longer DFS ($P = 0.003$, $P = 0.014$) and OS (VEGFR-2 −906, $P = 0.011$). CXCR-2 +1208 T/T genotype was a negative predictor for DFS ($P < 0.0001$). Combined analysis for DFS and OS indicated that patients with the fewest number of favorable genotypes simultaneously present (VEGFR-2 −906 T/T, CXCR-2 +1208 C/T or C/C and PAR-1 −506 Del/Del) were at the highest risk for recurrence or death ($P < 0.0001$).

*Correspondence to: Mr Y. K. Vashist, Department of General, Visceral and Thoracic Surgery, University Medical Center of Hamburg-Eppendorf, Martinistr. 52, 20246 Hamburg, Germany. Tel: +49-40-7410-54403; Fax: +49-40-7410-44995; E-mail: vashist@uke.de

†Both authors contributed equally to this work.

Supplementary material:

Table 1. Five year follow-up program for colon cancer patients ≤ 75 years and all rectal cancer patients after complete resection.

	Serum	Radiologic examination		Endoscopic procedure	
Months after surgery	CEA	Liver[1]	Lungs[2]	Coloscopy	Proctoscopy[3]
1	x				
6	x	x	x		x
12	x	x	x		x
18	x	x	x		x
24	x	x	x		x
30	x	x	x		x
36	x	x	x		x
42	x				
48	x	x	x		x
54	x				
60	x	x	x	x	x

[1]Ultrasonography of liver (computed tomography after 2006) [2]Chest x-ray (computed tomography after 2006) [3]Only rectal cancer after low anterior resection

Table 2. Characteristics of the cohorts included in the different analyses.

	All patients	Analyses of prevalence of MSI	Prognostic analyses
Variables	*N* (%)	*N* (%)	*N* (%)
Age			
Median (range)	74,3 (18.8 – 96.6)	73.2 (29.9 – 94.5)	73,0 (29.9 – 94.5)
< 60 y	*202* (16)	*146* (18)	*111* (18)
60 – 70	*243* (19)	*164* (20)	*126* (21)
70 – 80	*466* (37)	*301* (37)	*236* (38)
> 80	*363* (28)	*194* (24)	*140* (23)
Sex			
Female	*674* (53)	*431* (54)	*321* (52)
Male	*600* (47)	*374* (46)	*292* (48)
Tumor location			
Proximal colon	*451* (35)	*327* (41)	*238* (39)
Distal colon	*374* (29)	*274* (34)	*198* (32)
Rectum	*389* (31)	*204* (25)	*177* (29)
Synchronous	*30* (2)	*0*	*0*
Unknown	*30* (2)	*0*	*0*
Stage			
I	*155* (12)	*118* (15)	*117* (19)
II	*382* (30)	*323* (40)	*291* (48)
III	*251* (20)	*210* (26)	*188* (31)
IV	*312* (25)	*154* (19)	*17* (3)
Unknown	*174* (14)	*0*	*0*
Total	*1274*	*805*	*613*

PAPER III

Mutations in *BRAF* and *KRAS* identify stage-specific subgroups of colon cancer patients with inferior prognosis

Submitted manuscript

Marianne A. Merok, Stine A. Danielsen, Matthias Kolberg, Marianne Guriby, Merete Hektoen, Mette Eknæs, Håvard E. Danielsen, Aud Svindland, Arild Nesbakken, Ragnhild A. Lothe

APPENDIX

Prognostic impact of genomic instability in colorectal cancer

(Short title: Genomic instability and prognosis in CRC)

Manuscript

Tarjei S. Hveem, Marianne A. Merok, Maria E. Pretorius, Marco Novelli, Mette S. Bævre Ole H. Sjo, Noah Clinch, Knut Liestøl, Aud Svindland, Ragnhild A. Lothe, Arild Nesbakken, Håvard E. Danielsen