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THE PROGNOSTIC AND PREDICTIVE VALUE OF SELECTED BIOMARKERS IN COLORECTAL CANCER

by

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To my loved ones.

ABSTRACT

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The prognostic and predictive value of selected biomarkers in colorectal cancer

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Tissue-based biomarkers are studied to receive information about the pathologic processes and cancer outcome, and to enable development of patient-tailored treatments. The aim of this study was to investigate the potential prognostic and/or predictive value of selected biomarkers in colorectal cancer (CRC).

Group IIA secretory phospholipase A2 (IIA PLA2) expression was assessed in 114 samples presenting different phases of human colorectal carcinogenesis. Securin, Ki-67, CD44 variant 6 (CD44v6), aldehyde dehydrogenase 1 (ALDH1) and β-catenin were studied in a material including 227 rectal carcinoma patients treated with short-course preoperative radiotherapy (RT), long-course preoperative (chemo)RT (CRT) or surgery only. Epidermal growth factor receptor *(EGFR)* gene copy number (GCN), its heterogeneity in CRC tissue, and association with response to EGFR-targeted antibodies cetuximab and panitumumab were analyzed in a cohort of 76 metastatic CRC.

IIA PLA2 expression was decreased in invasive carcinomas compared to adenomas, but did not relate to patient survival. High securin expression after long-course (C)RT and high ALDH1 expression in node-negative rectal cancer were independent adverse prognostic factors, ALDH1 specifically in patients treated with adjuvant chemotherapy. The lack of membranous CD44v6 in the rectal cancer invasive front associated with infiltrating growth pattern and the risk of disease recurrence. Heterogeneous *EGFR* GCN increase predicted benefit from EGFR-targeted antibodies, also in the chemorefractory patient population.

In summary, high securin and ALDH1 protein expression independently relate to poor outcome in subgroups of rectal cancer patients, potentially because of resistance to conventional chemotherapeutics. Heterogeneous increase in *EGFR* GCN was validated to be a promising predictive factor in the treatment of metastatic CRC.

Keywords: biomarker, chemotherapy, colorectal cancer, EGFR-targeted antibody, predictive value, prognostic factor, radiotherapy, rectal cancer

TIIVISTELMÄ

Tuulia Avoranta

Valikoitujen merkkiaineiden ennusteellinen merkitys paksu- ja peräsuolisyövässä

Syöpätautien ja patologian oppiaineet, Turun Yliopisto; syöpäklinikka ja patologian yksikkö, Turun yliopistollinen keskussairaala; Turun Yliopiston tutkijakoulu; Valtakunnallinen kliininen tutkijakoulu

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Kudoslähtöisten merkkiaineiden tutkimisella toivotaan voivan saavuttaa lisätietoa syövän biologisesta käyttäytymisestä ja ennusteesta, sekä mahdollistavan kullekin potilaalle optimaalisen hoidon valinnan. Väitöstutkimuksen tavoitteena oli selvittää valikoitujen merkkiaineiden ennusteellista merkitystä paksu- ja peräsuolisyövässä.

Tyypin II sekretorisen fosfolipaasi A2:n (IIA PLA2) ilmentymistä tutkittiin 114:ssä syövän kehittymisen eri vaiheita edustavassa paksu- ja peräsuolikasvaimessa. Securinin, Ki-67:n, CD44 variantti 6:n (CD44v6), aldehydidehydrogenaasi 1:n (ALDH1) ja β-kateniinin ilmentymistä analysoitiin 227 peräsuolisyöpäpotilaan aineistossa, jossa oli mukana myös ennen leikkausta (kemo)sädehoidettuja potilaita. Levinnyttä paksu- tai peräsuolisyöpää sairastavien potilaiden (*n*=76) leikkausnäytteistä tutkittiin epidermaalisen kasvutekijäreseptorin *(EGFR)* geenikopiomäärää, sen kasvaimen sisäistä vaihtelua, sekä yhteyttä EGFR-vasta-ainehoidoista saavutettavaan hyötyyn.

IIA PLA2:n ilmentyminen syövässä oli vähäisempää kuin syövän esiasteissa. Securinin korkea ilmentyminen pitkän (kemo)sädehoidon jälkeen, sekä ALDH1:n korkea ilmentyminen imusolmukkeisiin leviämättömässä peräsuolisyövässä olivat itsenäisiä huonon ennusteen tekijöitä, ALDH1 etenkin solunsalpaajilla hoidetuilla potilailla. Heikentynyt solukalvon CD44v6-ilmentyminen oli yhteydessä syövän aggressiiviseen kasvutapaan ja peräsuolisyövän uusiutumisriskiin. Heterogeeninen *EGFR*-geenin kopiomäärän lisääntyminen ennusti hyvää vastetta EGFR-vasta-ainehoidoille levinneessä paksu- ja peräsuolisyövässä, mikä oli nähtävissä myös solunsalpaajahoidoille kehittyneen vastustuskyvyn jälkeisissä hoitolinjoissa.

Yhteenvetona todetaan, että korkea securinin ja ALDH1:n ilmentyminen peräsuolisyövässä ovat itsenäisiä huonon ennusteen merkkejä tietyissä alaryhmissä. Tämä voi heijastaa securin- ja ALDH1-positiivisten syöpäsolujen vastustuskykyä yleisesti käytetyille solunsalpaajille. Heterogeeninen *EGFR*-geenikopiomäärän lisääntyminen on lupaava tekijä ennustamaan EGFR-vasta-ainehoidoista saavutettavaa kliinistä hyötyä.

Avainsanat: EGFR-vasta-ainehoito, ennusteellinen merkkiaine, paksusuolisyöpä, peräsuolisyöpä, solunsalpaajahoito, sädehoito

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ABBREVIATIONS

5-FU	5-fluorouracil
IIA PLA2	Group IIA secretory phospholipase A2
ALDH1	Aldehyde dehydrogenase 1
ANOVA	Analysis of variance
APC	Adenomatous polyposis coli
APR	Abdominoperineal resection
CEA	Carcinoembryonic antigen
CD44v6	CD44 variant 6
CI	Confidence interval
CIN	Chromosomal instability
CIMP	CpG island methylator phenotype
CISH	Chromogenic *in situ* hybridization
CpG	Cytosine and guanine dinucleotides
CRC	Colorectal cancer
CRM	Circumferential margin
CRT	Chemoradiotherapy
CSC	Cancer stem cell
CT	Computed tomography
DFS	Disease-free survival time
DNA	Deoxyribonucleid acid
DSS	Disease-specific survival time
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
FAP	Familial adenomatous polyposis
FISH	Fluorescence *in situ* hybridization
FOB	Fecal occult blood
GCN	Gene copy number
Gy	Gray
HER2	Human epidermal growth factor receptor 2
ICC	Intra-class correlation coefficient
Ig	Immunoglobulin
ISH	*In situ* hybridization

kDa	Kilodalton
KRAS	Kirsten ras
LAR	Low anterior resection
LR	Likelihood ratio
LS	Lynch syndrome
MAPK	Mitogen-activated protein kinase
MMR	Mismatch repair
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleid acid
MSI	Microsatellite instability
MSI-H	High-level microsatellite instability
MSI-L	Low-level microsatellite instability
MSS	Microsatellite stable
NSAID	Non-steroidal anti-inflammatory drug
OS	Overall survival
pCR	Pathologic complete response
PET	Positron emission tomography
PFS	Progression-free survival
PI3K	Phosphatidylinositol 3-kinase
PLA2	Phospholipase A2
PTTG	Pituitary-tumor transforming gene
RR	Response rate
RT	Radiotherapy
SISH	Silver-enhanced *in situ* hybridization
sPLA2	Secretory phospholipase A2
SSA	Sessile serrated adenoma
TMA	Tissue microarray
TME	Total mesorectal excision
TNM	Tumor node metastases system
TRG	Tumor regression grade
TS	Thymidylate synthase
VEGF	Vascular endothelial growth factor
WT	Wild-type

LIST OF ORIGINAL PUBLICATIONS

I Avoranta T, Sundström J, Korkeila E, Syrjänen K, Pyrhönen S, Laine J. The expression and distribution of group IIA phospholipase A2 in human colorectal tumours. *Virchows Archiv* 2010; 457: 659 – 667

II Avoranta T, Korkeila E, Minn H, Syrjänen K, Pyrhönen S, Sundström J. Securin identifies a subgroup of patients with poor outcome in rectal cancer treated with long-course radiotherapy. *Acta Oncol* 2011; 50: 1158 – 66

III Avoranta T, Korkeila E, Syrjänen K, Pyrhönen S, Sundström J. Lack of CD44 variant 6 expression in rectal cancer invasive front associates with early recurrence. *World J Gastroenterol* 2012; 18: 459 – 56

IV Avoranta ST, Korkeila EA, Ristamäki RH, Syrjänen KJ, Carpén OM, Pyrhönen SO, Sundström JT. ALDH1 expression indicates chemotherapy resistance and poor outcome in node-negative rectal cancer. *Hum Pathol* 2013; doi: 10.1016/j.humpath.2012.10.003

V Ålgars A, Avoranta T, Österlund P, Lintunen M, Sundström J, Jokilehto T, Ristimäki A, Ristamäki R, Carpén O. Heterogenous *EGFR* gene copy number increase is common in colorectal cancer and defines response to anti-EGFR therapy. *Submitted*

1. INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies worldwide (Ferlay *et al.* 2010). Although the present-day surgical and oncologic treatments, including the preoperative (chemo)radiotherapy (CRT) of locally advanced rectal cancer, have improved patient survival, the predictability of disease progression is still challenging. This may be partly explained by a significant inter- and intratumoral heterogeneity. Accordingly, some of the high risk patients in a need of more aggressive treatment approaches may be escaped, while others are distressed with too heavy therapies. In the era of personalized medicine, tissue-based biomarkers have been studied to better predict treatment response and disease outcome, as well as to minimize harmful side-effects and economical burden caused by treatments.

Tumor cells utilize several methods to enlarge bulk tumor mass and to invade during disease progression, such as immunological mechanisms, accelerated cell proliferation, and altered adhesion to surrounding environment. Secretory phospholipase A2 group IIA (IIA PLA2) is an important inflammatory mediator, and has been related to adenoma-carcinoma sequence of CRC. However, the data on its expression in CRC is not unanimous (Edhemovic *et al*. 2001; Wendum *et al.* 2003; Buhmeida *et al.* 2009), rendering it an interesting target to study. The traditional proliferation marker Ki-67 has been studied in abundance to assess the association of proliferation with CRC outcome, but similarly to IIA PLA2, with inconclusive results (Brown and Gatter, 2002). Securin is another proliferation-associated marker that, unlike Ki-67, is involved in various other mechanisms relating to tumor progression as well (Pei and Melmed, 1997; Yu *et al.* 2000; Malik and Kakar, 2006). The data concerning its association with rectal cancer response to radiotherapy (RT) and outcome is incomplete. In addition to immunological mechanisms and cell proliferation, cancer cells differ from normal cells in their capability to adhere to extracellular matrix (ECM) and other cells. CD44 variant 6 (CD44v6) is an adhesion molecule related to CRC progression with highly conflicting prognostic value (Mulder *et al.* 1994; Morrin and Delaney, 2002; Zlobec *et al.* 2009).

According to cancer stem cell (CSC) hypothesis, only a small fraction of cancer cells are able to initiate and sustain malignant growth (Reya *et al.* 2001). The presence of these cells may jeopardize the antitumoral activity of oncologic treatments because of their intrinsic chemo- and radioresistance (Rich 2007; Dylla *et al.* 2008). Aldehyde dehydrogenase 1 (ALDH1) is one of the putative CSC markers and has been documented to increase during CRC progression (Huang *et al.* 2009), but there are few data on its actual prognostic and predictive value in rectal cancer.

Tumor heterogeneity complicates individualized treatment of cancer because of considerable variation in mutation and gene expression profile within a single tumor (Gerlinger *et al.* 2012). Cetuximab and panitumumab are monoclonal antibodies utilized in the treatment of metastatic CRC. They target epidermal growth factor receptor (EGFR)

that contributes to several tumor-promoting mechanisms. Mutations in *Kirsten ras* (*KRAS*) and other downstream molecules of EGFR signaling are important mechanisms of resistance to these therapeutic agents, but additional predictive factors are urgently needed to distinguish between responsive and nonresponsive patients. (Bardelli and Siena, 2010). The analysis of *EGFR* gene copy number (GCN) has been suggested to meet this request (Ålgars *et al.* 2011), but the results need to be further validated.

2. REVIEW OF THE LITERATURE

2.1. Epidemiology of colorectal cancer

2.1.1. Incidence and mortality

With an incidence of 1.23 million new cases per year, CRC is the third most commonly diagnosed cancer worldwide. Among men, it is the third, and among women the second most common cancer type (Ferlay *et al.* 2010). The median age at diagnosis is 69 years (Howlader *et al.* 2012). Men are at substantially higher risk of developing CRC as compared to women (overall sex ratio of the age-standardized ratios is 1.4:1). There is wide variation in incidence according to geographical position, the highest rates being estimated in Australia, New Zealand and Western Europe, and the lowest in Africa and South-Central Asia. (Ferlay *et al.* 2010.) The incidence rates have been falling or stabilizing in many countries where rates were previously high. In contrast, increasing rates are seen in economically transitioning countries along with westernalization. (Hamilton *et al.* 2010.) Estimated death rate of 608 000 (8% of all cancer deaths) renders CRC the fourth most common cause of death from cancer (Ferlay *et al.* 2010).

In Finland, the incidence of CRC was approximately 1400 among men and 1300 among women in 2010, rendering it the third and second most commonly diagnosed cancer in sexes, respectively. With over 1100 deaths per year, CRC is the second most common cause of death from cancer in Finland. (Finnish Cancer Registry.)

2.1.2. Tumor sites

Colon and rectal cancer are usually combined in cancer statistics although they are suggested to be distinct clinicopathologic entities (Li and Lai, 2009; Greystoke and Mullamitha, 2012), as depicted in **Table 1**. The location of CRC cases has been shown to be approximately as follows: 40% of cases in the proximal colon (cecum, ascending and transverse colon), 30% in the distal colon (descending and sigmoid colon), and 30% in the rectum (Ward *et al.* 2007). A shift from distal to proximal tumors over the decades has been observed in some studies (Li and Lai, 2009). In Finland, rectal cancer cases constituted 36% of new CRC cases in 2010 (Finnish Cancer Registry).

Table 1. Differences between proximal colon, distal colon, rectum, and carcinomas arising from them.

Feature	Proximal colon (prox. to splenic flexure)	Distal colon (distal to splenic flexure)	Rectum
Physiology			
•Embryological origin	Midgut	Hindgut	Hindgut
•Location	Peritoneal cavity	Peritoneal cavity	Mostly outside the peritoneal cavity
•Arterial supply	Superior mesenteric	Inferior mesenteric	Inferior mesenteric
•Major physical function	Water and salt absorption	Water and salt absorption	Fecal storage
Carcinogenesis			
•Molecular mechanism	MSI common	CIN predominates	CIN predominates
•Hereditary disease prominence	Lynch syndrome	FAP	FAP
•Aneuploidy	Common	Very common	Very common
•*KRAS* mutation	Very common	Common	Common
•*BRAF* mutation	Common	Rare	Rare
Clinicopathologic features			
•Age predominance	Older patients	No predominance	Younger patients
•Sex predominance	Females	Males	Males
•Mucinous histology	Common	Less common	Rare
•Appearance	Exophytic	Endophytic/annular	Endophytic/annular
Risks and protection			
•Overweight	Increased risk	Increased risk	No association
•Physical activity	Protective effect	Protective effect	No association

Modified based on Kouri, 1993; Li and Lai 2009; Hamilton *et al.* 2010; and Greystoke and Mullamitha, 2012. *Abbreviations:* CIN, chromosomal instability; FAP, familial adenomatous polyposis; MSI, microsatellite instability

2.2. Etiology of colorectal cancer

Approximately 75% of CRC cases are sporadic in nature, indicating that there is no inherited mutation predisposing to CRC or a familial history of CRC. Familial clustering of CRC is seen in approximately 25% of CRC cases. In 4/5 of these cases patient has an affected first degree relative, but the criteria for hereditary CRC are not fulfilled (termed as familial CRC in future). Hereditary CRC accounts for approximately 5% of all CRC cases, and includes the familial adenomatous polyposis (FAP) and Lynch syndromes (LS) with autosomal dominant inheritance of high-penetrance mutations. (de la Chapelle, 2004.)

CRC is a heterogeneous disease with at least three possible molecular pathways leading to its onset. The major chromosomal instability (CIN) pathway accounts for

most of the sporadic and familial cases, as well as for the CRC resulting from FAP syndrome. The 5'-CG-3' (cytosine and guanine dinucleotides, CpG) island methylator phenotype (CIMP) pathway accounts for significantly less of the sporadic and familial cases than the CIN pathway. The pure microsatellite instability (MSI) pathway results from a germline mutation in a deoxyribonucleic acid (DNA) mismatch repair (MMR) machinery, and is responsible for the LS. Regardless of the underlying pathway, several and sequential genetic alterations occur before a clinical presentation of CRC. (Haydon and Jass, 2002; Markowitz and Bertagnolli, 2009.) It has been suggested that a small population of stem cells could be the target of these alterations, leading to cellular transformation and subsequent tumorigenesis (Reya *et al*. 2001).

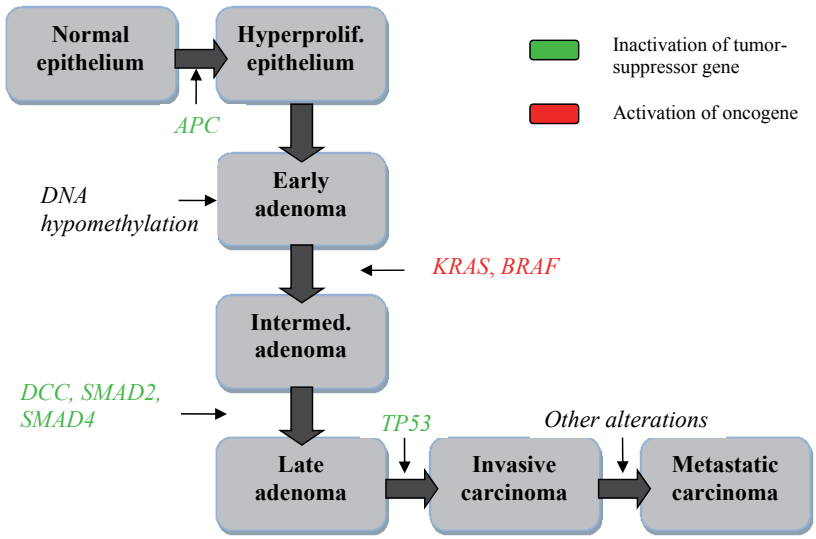

Figure 1. Colorectal carcinogenesis in CIN pathway. Modified from Fearon and Vogelstein, 1990; and Markowitz and Bertagnolli, 2009.

2.2.1. Chromosomal instability (CIN) pathway

Usually it takes 10-20 years for a normal colorectal epithelium to undergo molecular changes and develop into carcinoma (Tanaka, 2009). Several sequential inactivations of tumor suppressor genes and activating mutations of oncogenes take place, resulting in an altered structure of normal colorectal epithelium (Fearon and Vogelstein, 1990). Normal colorectal epithelium consists of cells that are arranged in straight tubular glands, crypts. Aberrant crypt foci are characterized by altered luminal openings, thickened epithelia and being larger than the adjacent normal crypts (Bird *et al.* 1987). They are postulated to be the earliest identifiable precursos of CRC (Bird *et al.* 1987), and are followed by the formation of premalignant adenomas (Fearon and Vogelstein, 1990) that are defined by the presence of dysplastic epithelium (Hamilton *et al*. 2010).

The cascade of colorectal carcinogenesis is presented in **Figure 1.** However, only a minority of CRCs characterized by CIN present with all the described mutations, and the accumulation of mutations is probably more important than their actual order (Fearon and Vogelstein, 1990). The earliest molecular alteration in colorectal carcinogenesis is the inactivating mutation of the adenomatous polyposis coli (*APC)* tumor-suppressor gene and/or loss of chromosome 5q including this gene (Fearon and Vogelstein, 1990; Miyoshi *et al.* 1992). This leads to accumulation of β-catenin, and activation of Wnt-signaling pathway that regulates among others growth, apoptosis, and maintenance of stem cell compartments (Korinek *et al.* 1997; Reya *et al.* 2001). Somatic mutations of the *APC* gene occur in 60-80% of the adenomas and sporadic CRC cases (Miyoshi *et al.* 1992). Other important impairments of tumor-suppressor genes include those of *DCC*, *SMAD2*, *SMAD4* and *TP53* resulting from the allelic losses of chromosome 18q and 17p, or decreased expression of their gene products, respectively. As in the case of *APC*, losses of chromosomes 17p and 18q are rather frequent events, but take place later during the cascade. A mutation or loss of heterozygosity of the *TP53* gene is considered as the most important point determining the conversion of an adenoma into a carcinoma. (Fearon and Vogelstein, 1990.)

Of the several oncogenes that play key roles in colorectal carcinogenesis, oncogenic mutations of *RAS* (mainly *KRAS*), *BRAF* and *PTEN* are the best characterized (Fearon and Vogelstein, 1990; Markowitz and Bertagnolli, 2009). They are present in about 40%, 10% and 10-15% of CRCs, respectively (Lièvre *et al.* 2006; Markowitz and Bertagnolli, 2009; Bardelli and Siena, 2010). As a result of their mutation, the mitogen-activated protein kinase (MAPK) and/or phosphatidylinositol 3-kinase (PI3K) signaling pathways may become constantly active, leading to an increased transcription of genes affecting growth, proliferation, and suppression of apoptosis. (Markowitz and Bertagnolli, 2009.) The mutations in *RAS, BRAF* and *PTEN* are considered as to account for the conversion of a small adenoma into a larger and more dysplastic one (Fearon and Vogelstein, 1990).

2.2.1.1. Familial adenomatous polyposis (FAP)

FAP, accounting for less than 1% of all CRC cases, is an autosomal dominant inherited disorder with a germline mutation in the *APC* gene. Other syndromes of the same disease spectrum include Gardner syndrome, attenuated adenomatous polyposis coli, and MYH-associated polyposis (de la Chapelle, 2004; Lynch *et al.* 2008). Colorectal carcinogenesis in FAP patients follows the principles of the CIN pathway described above (Fearon and Vogelstein, 1990). However, FAP patients are born with one mutated copy of the *APC* gene, and somatic inactivation of the other copy leads to adenoma initiation at relatively early age (Ichii *et al.* 1992). Hundreds to thousands adenomas may be detected from FAP patients usually since late childhood or adolescence, and if not adequately treated, most of the affected individuals will develop CRC by the age of 40 (de la Chapelle 2004; Lynch *et al.* 2008). FAP patients often have benign and malignant extracolonic manifestations, such as upper gastrointestinal polyps and thyroid cancer (Anaya *et al.* 2008). Genetic testing for FAP is offered at childhood when the specific *APC* mutation

has been identified in the family, and ascertained individuals require regular endoscopic surveillance from the age of 12-14. Prophylactic colectomy or proctocolectomy is considered as the standard treatment for FAP patients. (Balmaña *et al.* 2010.)

2.2.2. Microsatellite instability (MSI) pathway

MSI pathway is another important type of genetic instability in CRC. Microsatellites are short sequences of DNA repeated in tandem, and are found throughout the genome. They are prone to replication errors, and a defect of MMR proteins in correcting these errors leads to MSI. The MMR system is composed of at least eight proteins; MLH1, MLH3, MSH2, MSH3, MSH6, PMS1, PMS2 and Exo1. (Al-Sohaily *et al.* 2012.) There are several genes, such as transforming growth factor β (TGF-β) type II receptor, that are prone to mutations under MSI and, accordingly, are expressed inadequately (Alhopuro *et al.* 2012). Microsatellite status is divided into three groups by testing five microsatellites: microsatellite stable (MSS) if no instability is detected, low-level instability (MSI-L) if only one locus shows instability, and high-level instability (MSI-H) if two or more of the five loci show instability (Hamilton *et al.* 2010).

Approximately 10-15% of sporadic CRCs are MSI-H. In these cases, MSI is usually caused by epigenetic MLH1 promoter methylation, leading to loss of MLH1 expression. (Mäkinen, 2007.) Sporadic MSI-H CRCs differ from MSS and MSI-L CRC with respect to their mutational profile and clinicopathologic performances. They frequently harbor *BRAF* mutations, but rarely *KRAS* mutations (Hamilton *et al.* 2010). They are more likely to affect women, to arise in the proximal colon, to have an associated lymphocytic infiltrate, to produce excess mucin, and to be poorly differentiated (Jernvall *et al.* 1999; Mäkinen, 2007; Al-Sohaily *et al.* 2012). A substantial survival advantage in MSI-H CRC has been demonstrated (Sargent *et al.* 2010). The precursor lesion for sporadic MSI-H CRC is likely to be a sessile serrated adenoma (SSA) instead of conventional polypoid adenoma (Hawkins and Ward, 2001), accounting for up to 17.5% of proximal CRCs (Mäkinen, 2007). This view is supported by the resemblance of SSAs with MSI-H sporadic CRC. SSAs often present with deficient or absent function of MLH1 protein, frequently harbor *BRAF* mutations, and have predilection for the proximal colon. (Mäkinen, 2007.) SSAs belong to a group of serrated polyps together with hyperplastic polyps and traditional serrated adenomas. They are characterized by abnormal proliferation and distorted crypts, with closer resemblance to hyperplastic polyps than traditional serrated adenomas. (Snover *et al.* 2010).

2.2.2.1. Lynch syndrome

Lynch syndrome, also known as hereditary nonpolyposis colorectal cancer, accounts for 2-4% of the total CRC cases. As FAP, it is inherited in an autosomal dominant manner, but the germline mutation responsible for increased risk of CRC is in one of the MMR genes, most commonly in *MLH1* or *MSH2*. (Kinzler and Vogelstein, 1996.) Only one somatic inactivation of the wild-type (WT) parental allele is thus needed to silence the

gene function leading to MSI, accelerated adenoma-carcinoma sequence, and a lifetime risk of CRC of about 80% (de la Chapelle, 2004). *BRAF* mutation is hardly ever seen in LS-associated cancers (Hamilton *et al.* 2010). The average age of CRC onset in LS patients is 45-60 years. Otherwise, the tumors of LS patients show special pathologic features similar to sporadic MSI-H CRC. (Lynch *et al*. 2008.) Patients also are at increased risk for other malignancies, among others gynecologic, urothelial, gastric, small bowel and brain tumors (Anaya *et al.* 2008). Both the Amsterdam II criteria (Vasen *et al.* 1999) and the revised Bethesda guidelines (Umar *et al.* 2004) presented in **Table 2** are utilized to identify individuals with suspicion of LS or candidates for molecular screening. Endoscopic surveillance is recommended for patients from the age of 20-25 years, and endometrial and ovarian cancer screening from the age of 30-35 years (Balmaña *et al.* 2010). Surveillance cannot compensate for the increased risk of CRC, but decreases the cancer-specific mortality of mutation carriers (Järvinen *et al*. 2009). The precursor lesion in LS is usually an adenoma, and removal of adenomas decreases the risk of CRC in LS patients (Kinzler and Vogelstein, 1996; Mecklin *et al*. 2007). Prophylactic colectomy is considered in selected cases (Lynch *et al*. 2008).

Table 2. Guidelines for identifying Lynch syndrome.

Amsterdam II criteria (Vasen *et al.* 1999). Each of the criteria should be fulfilled.
1. At least three relatives with LS-associated cancer (CRC, cancer of the endometrium, small bowel, ureter, or renal pelvis)
2. One should be a first-degree relative of the first two
3. At least two successive generations are affected
4. At least one should be diagnosed before age 50
5. FAP has been excluded
6. Tumors should be verified by pathological examination
Revised Bethesda guidelines (Umar *et al.* 2004). At least one criterion should be fulfilled.
1. CRC diagnosed in a patient who is younger than 50 years of age
2. Presence of synchronous, metachronous colorectal, or other LS-associated tumors, regardless of age
3. Colorectal cancer with MSI-H histology diagnosed in a patient who is younger than 60 years of age
4. CRC diagnosed in at least one first degree relatives with a LS-associated tumor, with one of the cancers being diagnosed under age of 50 years
5. CRC diagnosed in at least two first or second degree relatives with LS-associated tumor, regardless of age

Adopted from Vasen *et al.* 1999; and Umar *et al.* 2004. *Abbreviations:* CRC, colorectal cancer; FAP, familial adenomatous polyposis; LS, Lynch syndrome; MSI-H, high-level microsatellite instability

2.2.3. CpG island methylator (CIMP) pathway

Epigenetic alterations refer to changes in gene expression or function caused by other than changes in the underlying DNA sequences. DNA methylation, commonly at the

CpG-nucleotides, is the most usual epigenetic change in human, and may result in inactivation of genes such as *APC*, *MLH1*, and several others. CIMP-high CRC accounts for 15-20% of sporadic CRC, and is the mechanism underlying most of the sporadic CRC cases with MSI. (Al-Sohaily *et al.* 2012.) On the other hand, over 50% of CIMP carcinomas are MSS. Mutations in *BRAF*, *KRAS*, and *TP53* are frequent in tumors that have arisen along CIMP pathway (Weisenberger *et al.* 2006; Hamilton *et al.* 2010.) It has been proposed that in MSI-H CIMP carcinomas, the precursor lesion is SSA (Mäkinen, 2007) in contrast to adenomatous polyps in CIN and pure MSI pathways (Al-Sohaily *et al.* 2012). Instead, the precursor lesion in MSI-L/MSS CIMP carcinoma might be a traditional serrated adenoma (Mäkinen, 2007).

2.2.4. Other predisposing conditions and risk factors

The risk of CRC increases along with age. The risk is higher in males compared to females, and in African-American population compared to people of other races. (Howlader *et al.* 2012.) The individuals with a history of previous adenoma or CRC, and those with RT in the pelvic area are at increased risk of CRC (Baxter *et al.* 2005; Mysliwiec *et al.* 2006), as are the patients with colitis ulcerosa or Crohn's disease (Eaden *et al.* 2001).

2.2.4.1. Hamartomatous polyposis syndromes

Peutz-Jeghers syndrome, Juvenile polyposis syndrome, and Cowden syndrome represent a group of rare inherited disorders with relatively benign appearing polyps of the gastrointestinal tract. The affected individuals are at increased risk of CRC, as well as of other gastrointestinal and extra-gastrointestinal malignancies. (Lynch *et al.* 2008.)

2.2.4.2. Microbial and viral pathogens

In contrast to some other cancer types, no direct causal link has been confirmed between microbial infection and CRC. However, several pathogens such as *Streptococcus Bovis*, *Helicobacter Pylori*, *Escherichia Coli* and human papillomavirus have been related to colorectal carcinogenesis. The mechanisms for oncogenic potential have been suggested to include inactivation of tumor-suppressor genes, induction of inflammation, and production of mutagenic toxins, thus contributing to pro-carcinogenic environment. (Collins *et al*. 2011.)

2.2.4.3. Nutrition and environment

Overall, up to 55% of CRC burden is estimated to link with environmental factors (Parkin *et al.* 2011). The risk has constantly been related to western-type of diet (Aune *et al.* 2011; Parkin *et al.* 2011), although wide heterogeneity exists between studies concerning risk factors. High consumption of red and processed meat is rather convincingly related to increased risk of both colon and rectal cancer (Chan *et al.* 2011). Smoking exposure increases the risk of adenomatous and serrated polyps (Ji *et al.* 2006), and relates to increased risk of rectal cancer especially (Liang *et al.* 2009). Alcohol intake is associated

with increased risk of both cancer types (Fedirko *et al.* 2011). Overweight is associated with increased risk of colon cancer among men, while the association is weaker among women and in rectal cancer (Renehan *et al.* 2008).

2.2.5. Prevention

The use of non-steroidal anti-inflammatory drugs (NSAIDs), mainly aspirin, has been shown to decrease the risk of colorectal adenomas and carcinomas both in hereditary and sporadic setting (Giardiello *et al.* 1993; Cole *et al.* 2009; Rothwell *et al.* 2012). The development of distant metastases and the mortality due to CRC have also been reported to reduce in aspirin users versus non-users (Rothwell *et al.* 2012a; Rothwell *et al.* 2012b), although the optimal treatment dose and duration are not fully established (Garcia-Albeniz *et al.* 2011). Not all studies, however, have demonstrated such an effect (Cook *et al.* 2005), and the known adverse effects of aspirin and other NSAIDs have limited their widespread use in chemoprevention. The benefits of aspirin use are most likely to overcome the risks in individuals at high risk for CRC, such as those with inherited syndromes or a history of previous CRC (Garcia-Albeniz *et al.* 2011).

The protective role of oral estrogen against CRC is incongruent (Bosetti *et al.* 2009; Long *et al.* 2010). With regard to nutritional and lifestyle factors, high-fiber diet (Aune *et al.* 2011) and the intake of calcium, milk and garlic may reduce the risk of CRC (World Cancer Research Fund, 2011). The evidence suggesting the protective effect of foods containing folate, selenium and vitamin D, as well as diet rich in fish, fruits and vegetables, is less convincing (Huxley *et al.* 2009; World Cancer Research Fund, 2011). High level of physical activity may reduce the risk of colon cancer, but not that of rectal cancer (Wolin *et al*. 2009).

2.3. Diagnosis and staging of colorectal cancer

2.3.1. Screening

The goal of screening for CRC is to reduce mortality from CRC by identification and removal of premalignant adenomas and/or early-stage carcinomas. Screening programmes base on detection of blood from stools (FOB, fecal occult blood), or visual inspection of the colon and rectum with endoscopy (Sigurdsson *et al.* 2012). In the screened population, a reduction in CRC mortality (Sigurdsson *et al.* 2012), and incidence in some studies (Atkin *et al.* 2010), has been reported, but not without concerns about specificity and sensitivity of the screening tests. In the Europe, immunochemical FOB test is currently recommended as the test of choice for population screening (Halloran *et al.* 2010). It is more sensitive than the conventional guaic-based tests but, simultaneously, less specific at the population level (Malila 2012, personal communication). In case of a positive test result, individuals should be referred for full colonoscopy (Halloran *et al.* 2010).

In 2004, Finland started a population-based screening programme for individuals aged 60-69 years, using a biannual guaic-based FOB test. The programme has been gradually expanding, and covered approximately 40% of the target population by the end of 2008. Attendance rates have been reasonably high (close to 70%), and screening has been shown to increase the detection of early CRC cases. (Paimela *et al.* 2010; Malila *et al*. 2011.)

2.3.2. Signs and symptoms

A change in bowel habits is a common symptom in CRC patients. It is more common in tumors of the distal colon than in those of the proximal colon, and includes diarrhea, constipation, change in the consistency of the stools and/or a feeling of incomplete emptying of the bowel. Unexplained weight loss and abdominal discomfort or pain, which is severe in the case of total bowel obstruction, may also be early symptoms. Tumor hemorrhage may result in rectal bleeding, blood in stools and iron deficiency with subsequent anemia, fatigue and weakness. (Hamilton and Sharp, 2004.) In the case of rectal cancer, a palpable mass may be detected in digital rectal examination in over 50% of the cases (Lepistö *et al.* 2009).

2.3.3. Diagnostic procedures and clinical staging

CRC diagnosis is based on endoscopic examination of the bowel with sigmoidoscopy or colonoscopy, and histological confirmation of the malignant tumor. Further staging is mandatory to specify disease prognosis, and to choose a suitable treatment strategy for a patient. (Schmoll *et al.* 2012). Staging is described according to TNM (tumor, node, metastases), Dukes or Astler-Coller systems. The assessment of the extent of CRC is presented in **Figure 2**, and the staging according to TNM7 in **Tables 3a** and **3b.** The previous version of the TNM staging system is depicted on **page 54**. The stage distribution at the time of diagnosis is as follows: 39% for localized stage I-II disease, 36% for regional stage III disease, 20% for distant stage IV disease, and 5% for unstaged disease, respectively (Howlader *et al.* 2012). Clinical staging, denoted as cTNM, is based on the information obtained before the surgical treatment of the tumor and includes (Schmoll *et al.* 2012):

- Physical examination including digital rectal examination and the assessment of performance status,
- Total colonoscopy (if not done) to find possible synchronous lesions,
- Carcinoembryonic antigen (CEA) determination,
- Computed tomography (CT) scans of the chest, abdomen and pelvis to find possible metastases,

- Endorectal ultrasound or pelvic magnetic resonance imaging (MRI) of the rectum in rectal cancer staging, and
- Positron emission tomography (PET) together with CT (PET-CT) may sometimes be essential to define metastatic spread

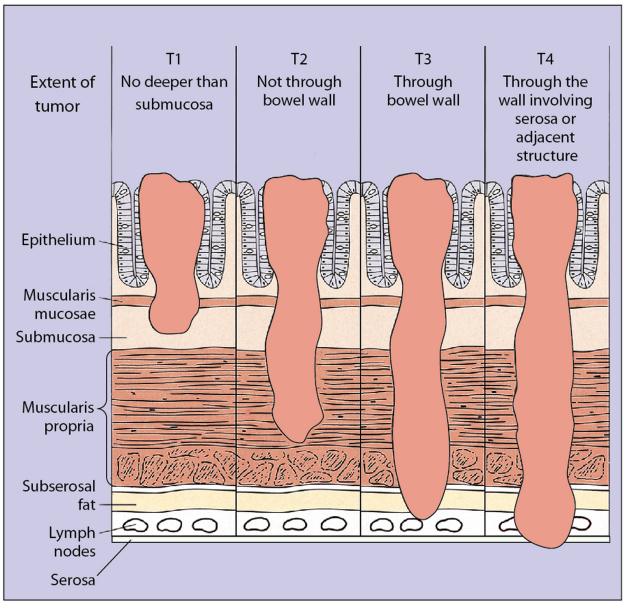

Figure 2. T-categories of colorectal cancer. Modified from Abeloff MD, Armitage JO, Niederhuber JE, Kastan MB, McKenna WG: Abeloff's Clinical Oncology, Chapter: Colon cancer, page 1496.

2.3.3.1. Imaging modalities in rectal cancer staging

Endorectal ultrasound performs well in differentiating superficial (T1 and T2) rectal tumors from those with deeper ingrowth into the rectal wall (T3 and T4), but is not as accurate as MRI in staging T3 and T4 tumors. MRI, in turn, is less accurate in differentiating T1 tumors from T2 tumors because of its inability to visualize submucosal and mucosal layers separately. (Valentini *et al.* 2008; Siegel *et al.* 2011.) The advantages of MRI include its accuracy in depicting the extramural depth of tumor invasion (MERCURY Study Group, 2007), and assessing the involvement of circumferential resection margin (CRM) between the edge of the tumor and the rectal fascia (Lahaye *et al.* 2005). It can also give information about the presence of extramural venous invasion (Brown *et al.* 2003a), and is not as operator-dependent as ultrasound (Valentini *et al.* 2008). MRI has become the predominant diagnostic imaging tool in rectal cancer staging (Schmoll *et al*. 2012).

The assessment of nodal status is extremely important in rectal cancer staging. So far, none of the imaging modalities has been proved superior to another with this respect (Lahaye *et al.* 2005). It is well established that also small lymph nodes may harbor a metastasis, and that the information about signal intensity together with lymph node border contour are more accurate in nodal staging (Brown *et al,* 2003b). Recently, MRI with lymph node-specific nanoparticle contrast mediums has been reported to be

promising in distinguishing between node-negative (N0) and node-positive (N1 and N2) patients (Lahaye *et al.* 2008). Lahaye with his colleagues presented that analyzing the percentage of the area with increased signal intensity after contrast agent administration has good specificity and sensitivity in dinstinguishing malignant lymph nodes from benign ones (Lahaye *et al.* 2008).

Fluorodeoxygluocose-PET/CT and diffuse-weighted MRI are imaging modalities that enable observation of the functional and molecular processes of the tumor. They have been of interest when developing methods for early prediction and assessment of radiotherapy (RT) response in rectal cancer. Recently, when combined at different time points, these imaging tools were shown to be promising in the response predicition and assessment (Lambrecht *et al*. 2010).

2.3.3.2. The role of pathology in diagnosis

In the diagnostic phase, the role of pathology is to classify the lesions sampled during endoscopy. Regarding non-cancerous lesions, the size, histological type (tubular, villous, tubulo-villous adenoma; serrated lesions; other polyps), grade of dysplasia (low-grade, high-grade), and the absence of submucosal invasion should be stated (Hamilton *et al*. 2010; Quirke *et al.* 2011). Adenomas are characterized by enlarged, hyperchromatic nuclei, varying degrees of nuclear spindling and stratification, and loss of polarity, but without invasion through the muscularis mucosae (Hamilton *et al.* 2010). When submucosal invasion is detected, the lesion is by definition malignant, and additional surgery is needed if unfavourable histopathologic features are present (Quirke *et al.* 2011). These features include deep submucosal invasion, positive resection margin, poor differentiation grade, lymphovascular invasion, and tumor budding (Cooper, 2007).

In the case of carcinoma, tumor histological type and differentiation grade should be stated (Quirke *et al*. 2011). More than 90% of CRCs are adenocarcinomas (Hamilton *et al.* 2010). Conventional, i.e. not otherwise specified, adenocarcinoma is the most common histological type, followed by mucinous carcinoma, some less common types of adenocarcinoma (signet-ring cell, medullary, serrated, and papillary), and non-adenocarcinoma (Howlader *et al*. 2012). Mucinous carcinomas by definition have more than 50% of extracellular mucin (Hamilton *et al.* 2010). Compared to non-mucinous carcinomas, they are more often situated in the proximal colon, are more common in females, and present with more advanced disease stage. MSI, CIMP-phenotype, and *BRAF* mutations are more common in mucinous than in conventional adenocarcinoma. (Verhulst *et al.* 2012.)

Table 3a. TNM classification of colon and rectum carcinoma, 7th edition.

T – Primary Tumor	N – Regional Lymph Nodes
TX Primary tumor cannot be assessed **T0** No evidence of primary tumor **Tis**[1] Carcinoma in situ: intraepithelial or invasion of lamina propria **T1** Tumor invades submucosa **T2** Tumor invades muscularis propria **T3** Tumor invades subserosa or into non-peritonealized pericolic or perirectal tissues **T4** Tumor directly invades other organs or structures and/or perforates visceral peritoneum **T4a** Tumor perforates visceral peritoneum **T4b** Tumor directly invades other organs or structures [2,3]	**NX** Regional lymph nodes cannot be assessed **N0** No regional lymph node metastasis **N1** Metastasis in 1-3 regional lymph nodes **N1a** Metastasis in 1 regional lymph node **N1b** Metastasis in 2-3 regional lymph nodes **N1c** Tumor deposit(s), i.e. satellites*, in the subserosa, or in the non-peritonealized pericolic or perirectal soft tissue *without* regional lymph node metastasis **N2** Metastasis in 4 or more regional lymph nodes **N2a** Metastasis in 4-6 regional lymph nodes **N2b** Metastasis in 7 or more regional lymph nodes
Notes: 1. Tis includes cancer cells confined within the glandular basement membrane (intraepithelial) or mucosal lamina propria (intramucosal) with no extension through the muscularis mucosae into the submucosa. 2. Direct invasion in T4b includes invasion of other organs or segments of the colorectum by way of the serosa, as confirmed on microscopic examination, or for tumors in a retroperitoneal or subperitoneal location, direct invasion of other organs or structures by virtue of extension beyond the muscularis propria. 3. Tumor that is adherent to other organs or structures, macroscopically, is classified as cT4b. However, if no tumor is present in the adhesion, microscopically, the classification should be pT1-3, depending on the anatomical depth of invasion.	**Notes:** * Tumor deposits (satellites), i.e., macroscopic or microscopic nests or nodules, in the pericolorectal adipose tissue's lymph drainage area of a primary carcinoma without histological evidence of residual lymph node in the nodule, may represent discontinuous spread, venous invasion with extravascular spread (V1/2) or a totally replaced lymph node (N1/2). If such deposits are observed with lesions that would otherwise be classified as T1 or T2, then the T classification is not changed, but the nodule(s) is recorded as N1c. If a nodule is considered by the pathologist to be a totally replaced lymph node (generally having a smooth contour), it should be recorded as a positive lymph node and not as a satellite, and each nodule should be counted separately as a lymph node in the final pN determination.
M – Distant Metastasis	
M0 No distant metastasis **M1** Distant metastasis **M1a** Metastasis confined to one organ (liver,lung, ovary, non-regional lymph node(s)) **M1b** Metastasis in more than one organ or the peritoneum	

International Union Against Cancer, TNM classification of Malignant Tumors, 7th edition, 2010, Wiley-Blackwell, Singapore. Copyright (2013) with permission from Wiley & Sons, Ltd.

Table 3b. Stage grouping of colon and rectum carcinoma.

Stage 0	Tis	N0	M0
Stage I	T1, T2	N0	M0
Stage II	T3, T4	N0	M0
Stage IIA	T3	N0	M0
Stage IIB	T4a	N0	M0
Stage IIC	T4b	N0	M0
Stage III	Any T	N1, N2	M0
Stage IIIA	T1, T2	N1	M0
	T1	N2a	M0
Stage IIIB	T3, T4a	N1	M0
	T2, T3	N2a	M0
	T1, T2	N2b	M0
Stage IIIC	T4a	N2a	M0
	T3, T4a	N2b	M0
	T4b	N1, N2	M0
Stage IVA	Any T	Any N	M1a
Stage IVB	Any T	Any N	M1b

International Union Against Cancer, TNM classification of Malignant Tumors, 7th edition, 2010, Wiley-Blackwell, Singapore. Copyright (2013) with permission from Wiley & Sons, Ltd.

The determination of differentiation grade is shown in **Table 4**. It is of major importance in superficial tumors, as T1 tumors showing good or moderate differentiation may be considered eligible for local excision (Quirke *et al.* 2011).

Table 4. Criteria for histological grading of colorectal adenocarcinomas.

Criterion	Differentiation category	Numerical grade[a]	Descriptive grade
>95% with gland formation	Well-differentiated	1	Low
50-95% with gland formation	Moderately differentiated	2	Low
0-49% with gland formation	Poorly differentiated	3	High
High level of MSI[b]	Variable	Variable	Low

[a] The category "undifferentiated carcinoma" (grade 4) is reserved for carcinomas with no gland formation, mucin production, or neuroendocrine, squamous, or sarcomatoid differentiation; [b] Microsatellite instability.

According to WHO Classification of Tumours of the Digestive System, 4th edition, 2010, International Agency for Research on Cancer, Lyon, p 138.

2.4. Treatment of colorectal cancer

Approximately 75-80% of CRC cases present as stage I-III at the time of diagnosis (Howlader *et al*. 2012). Surgery is the only curative treatment, but even after initial R0-resection, a significant proportion of patients will develop disease recurrence. R0-resection indicates that no residual tumor is left behind, while in R1- and R2-resections (chapter *2.5.1*) microscopic or macroscopic residual tumor is detected, respectively (Hamilton *et al*. 2010). The treatment of local colon and rectal cancer are described separately due to specific features of rectal cancer treatment.

2.4.1. Treatment of local (stage I-III) colon cancer

2.4.1.1. Colon cancer surgery

The main surgical procedure in colon cancer is hemicolectomy with en-bloc removal of the arterial arcade containing the regional lymph nodes, and margins of at least 5 cm on either side of the tumor. Subsequent anastomosis of the remaining colon sections is performed. In expert hands, laparoscopy-assisted resection is an accepted treatment option for colon cancer that does not invade surrounding structures. (Schmoll *et al.* 2012.) It has shown to be comparable with open surgery in terms of oncologic outcome (COST Study Group, 2004; Jayne *et al*. 2010), and superior in terms of intraoperative blood loss, postoperative bowel function, and duration of hospital stay (Lacy *et al.* 2002).

2.4.1.2. Adjuvant chemotherapy in colon cancer

The aim of postoperative adjuvant chemotherapy is to eradicate possible micrometastases and to decrease the risk of recurrence (Schmoll *et al.* 2012). The cornerstone of adjuvant treatment for stage III colon cancer are 5-fluorouracil (5-FU) (Moertel *et al.* 1990) or its oral prodrug capecitabine (Twelves *et al.* 2005) together with oxaliplatin (André *et al*. 2004). After resection of the primary tumor, this combination reduces the risk of death by 20% as compared to 5-FU alone (André *et al.* 2009). In stage II disease, the efficacy of adjuvant chemotherapy is less evident, but eligible patients with a tumor presenting high-risk features are usually offered 5-FU or capecitabine, reducing the risk of death by absolute 3-5% (Gray *et al*. 2007). High-risk features include pathologic (p) T4 tumor, less than 12 examined lymph nodes, poor differentiation grade, vascular, lymphatic or perineural invasion, and clinical presentation with intestinal occlusion or perforation (Schmoll *et al.* 2012). The most common adverse effects of chemotherapy regimens are neutropenia, stomatitis, and diarrhea for 5-FU, hand-foot syndrome for capecitabine, and neuropathy for oxaliplatin (Meyerhardt and Mayer, 2005). Patients with increasing age and comorbidites are less often treated with adjuvant chemotherapy, although thay have been demonstrated to benefit from the treatment (Wildes *et al.* 2010). Regimens including irinotecan (Saltz *et al.* 2007), bevacizumab (Allegra *et al*. 2011) and cetuximab (Alberts *et al.* 2012) have been studied but are not recommended in adjuvant setting because of increased toxicity and/or lack of significant clinical benefit.

2.4.2. Treatment of local (stage I-III) rectal cancer

Determination of the treatment approach is especially challenging in the case of rectal cancer. Multidisciplinary team work is invaluable when the goal is to achieve optimal disease control with minimal impairment of quality of life. In the Hospital District of Southwest Finland, rectal cancer surgery has been centralized to one hospital since 2004, and postoperative treatment of the patients is discussed in a multidisciplinary team consisting of a surgeon, medical and radiation oncologist, radiologist, pathologist, and a representative of nursing personnel.

2.4.2.1. Preoperative (chemo)radiotherapy for rectal cancer

Local recurrence after initially curative resection is more common in rectal than in colon cancer (Galandiuk *et al.* 1992). It is difficult to cure and present with extremely troublesome symptoms to a patient. Aims of preoperative (chemo)radiotherapy are to decrease the risk of local recurrence, to improve R0-resection rates, and to enable operation with anal sphincter -saving procedures whenever possible (Schmoll *et al.* 2012). RT with (Gastrointestinal Study Group, 1985; Chari *et al.* 1995) or without (Fisher *et al.* 1988; Swedish Rectal Cancer Trial, 1997) concomitant chemotherapy decreases the risk of local recurrence in stage II and III rectal cancer as compared to surgery alone. Either pre- or postoperative strategies have been utilized, but preoperative one is favored because of its beneficial efficacy and toxicity profiles (Sauer *et al.* 2004; Sauer *et al.* 2012). In high rectal cancer (over 10 cm from the anal verge), preoperative treatment is not routinely recommended (Schmoll *et al.* 2012). The evolution of RT strategies is shown in **Table 5**.

RT is administered using three portal or four portal-box techniques to the area comprising the primary tumor with its adjacent lymph nodes, thus extending from the superior border of L5/S1 vertebrae to the inferior border of obturator foramen (Swedish Rectal Cancer Trial, 1997; Sauer *et al*. 2004). Improvements in radiation techniques together with smaller irradiated volumes have reduced the risk of adverse effects, but they still are of major concern in treatment of rectal cancer. Problems with wound healing, gastrointestinal, genitourinary, and neurological functions are common acute adverse effects of irradiation. The most common late adverse effects, occuring over six months after the start of irradiation include bowel obstruction, bowel dysfunction and sexual dysfunction. An increased risk of secondary cancers has also been reported in irradiated patients. (Birgisson *et al.* 2007.)

2.4.2.1.1. Short-course preoperative radiotherapy

Short-course RT is recommended for stage II and III tumors that do not present with a threat of CRM involvement in MRI (Schmoll *et al.* 2012). It consists of five 5-gray (Gy) fractions delivered during one week, and surgery on the following week (Swedish Rectal Cancer Trial, 1997). No tumor downstaging has been demonstrated following this conventional “immediate surgery” -strategy (Marijnen *et al.* 2001), whereas “delayed

surgery" (4-8 weeks after RT) -strategy may lead to downstaging (Pettersson *et al.* 2010). Short-course RT improves local disease control (Swedish Rectal Cancer Trial 1997) even in combination with total mesorectal excision (TME) surgery (Kapiteijn *et al.* 2001), with 2- and 6-year local recurrence rates of as low as 2% and 6%, respectively (Kapiteijn *et al.* 2001; van Gijn *et al.* 2011). After a median follow-up of 10 years, the local recurrence rate was 5% in irradiated patients and 11% in the surgery-only group (van Gijn *et al.* 2011). The benefit from RT seems to be higher for stage III than for stage II disease (van Gijn *et al.* 2011). The effect of short-course RT on overall survival (OS) remains inconclusive (Folkesson *et al.* 2005; van Gijn *et al.* 2011).

2.4.2.1.2. Long-course preoperative chemoradiotherapy (CRT)

Long-course preoperative chemoradiotherapy (CRT) is recommended for patients with tumors directly infiltrating to other organs (T4), or with a threat of CRM involvement in MRI. The aim is tumor downstaging to allow R0-resection, and accordingly, to decrease the risk of local recurrence. RT is given in 25-28 fractions, five days per week, to a total dose of 50.4 Gy, over a period of approximately five weeks. Operation is performed 4-8 weeks later. Concomitantly with RT, capecitabine or 5-FU is administrated as a radiosensitizer. (Schmoll *et al.* 2012.) Oral capecitabine has been demonstrated to be an effective and well tolerated alternative to intravenous 5-FU, and is widely preferred due to its convenient route of administration (Hofheinz *et al*. 2012).

Table 5. Evolution of radiotherapy strategies in treatment of rectal cancer.

Year	
1990	Postoperative CRT was recommended for patients with ≥ T3 or node-positive tumors (NIH consensus statement, 1990)
1997	Short-course 5x5 Gy preoperative RT plus surgery improved local control and OS compared to surgery alone (Swedish Rectal Cancer Trial, 1997)
2001	Short-course preoperative RT improved local control even when combined to TME surgery (Kapiteijn *et al.* 2001)
2004	Preoperative long-course CRT compared to RT alone: more toxic, higher rate of pCR, superior local control, no OS benefit (Bosset *et al.* 2005)
2004	Preoperative CRT compared to postoperative CRT: superior local control, less toxicity, no OS benefit (Sauer *et al*. 2004)
2006	Preoperative short-course RT compared to preoperative CRT: no differences in local control, survival, or toxicity (Bujko *et al.* 2006)
2009	Short-course RT is superior to selective postoperative CRT in terms of local control (Sebag-Montefiore *et al.* 2009)
2010	Lenghtening the interval between short-course RT and surgery may allow tumor donwnstaging (Pettersson *et al.* 2010)
2011	After 10-year follow-up, preoperative short-course RT reduced local recurrence by more than 50% relative to surgery alone, and improved 10-year survival in TNM stage III disease with negative CRM (van Gijn *et al.* 2011)

Abbreviations: CRM, circumferential resection margin; CRT, chemoradiotherapy; Gy, gray; OS, overall survival; pCR, pathologic complete response; RT, radiotherapy; TME, total mesorectal excision

Tumor downstaging is seen in approximately 60% (García-Aguilar *et al*. 2003) and pathologic complete response (pCR) in 0-27% of patients after CRT (Dworak *et al.* 1997; Janjan *et al.* 1999). The definite effect of preoperative CRT on the number of sphincter-saving procedures remains indecisive, partly because the surgical techniques have improved at the same time with preoperative treatment modalities (Valentini *et al.* 2008). Five-year local recurrence rates have been shown to be as low as 6% after CRT and surgery (Sauer *et al.* 2004), but OS has not improved compared to RT alone (Fiorica *et al.* 2010). Preoperative CRT is favored over postoperative treatment because of improved local control, reduced toxicity, and better compliance (Sauer *et al*. 2004).

Recently, the efficacy of CRT has been further attempted to improve by the concept of induction chemotherapy, by addition of biologic agents or other chemotherapeutic drugs to conventional 5-FU based regimen (Engels *et al.* 2012), and by exposing tumor tissue to a high-dose CRT of 60 Gy (Vestermark *et al.* 2008). However, these regimens are not recommended in preoperative setting outside clinical trials (Engels *et al.* 2012).

2.4.2.2. Rectal cancer surgery

The rectum is anatomically divided in low, mid and high rectum according to its distance from the anal verge. The challenges of rectal cancer surgery stem from the pelvic anatomy; rectum is situated deep in the pelvis, close to the mesorectal fascia and nerves supplying genitourinary functions (Heald and Ryall, 1986; Siegel *et al.* 2011). In expert hands and selected cases, laparoscopy-assisted surgery may be a feasible alternative for open surgery (Jayne *et al*. 2010; Siegel *et al.* 2011), but it is not recommended as a standard modality (Schmoll *et al.* 2012). Early stage (cT1-2, N0) rectal cancer may be operated without preceding RT. For cT1 tumor without any high risk features, a local transanal excision may be considered. In stage II-III rectal cancer, treatment with (chemo)RT is recommended prior to surgery. (Schmoll *et al.* 2012.) For tumors showing pCR after preoperative CRT, non-operative treatment approaches have been evaluated (Habr-Gama *et al.* 2004), but because of poor correlation between clinical and pathologic complete response (Nyasavajjala *et al.* 2010) non-operative treatment approaches are not recommended at the moment (Schmoll *et al.* 2012).

For mid and low rectal cancer not eligible for local excision, transabdominal resection with TME is performed. For high rectal cancer, partial mesorectal excision is considered as adequate. (Schmoll *et al.* 2012.) In TME, rectum and mesorectum are resected within an intact mesorectal fascia, thus removing tumor together with its lymphatic and venous drainage, leaving pelvic autonomic nerves intact (Heald and Ryall, 1986). The introduction of TME (Heald and Ryall, 1986) resulted in a decrease of local recurrence rates from up to 40% to under 10% (Lange *et al.* 2009). Sphincter preservation procedures are preferred whenever possible. Low anterior resection (LAR) with TME and a subsequent anastomosis may usually be performed if a distal margin of 4 to 5 cm can be achieved. In low rectal cancer (under five cm from the anal verge), distal margin of 1 to 2 cm is considered acceptable, but the risk of anastomotic leakage is increased, in which case a transient colostomy may

be constructed. If creation of an anastomosis is not possible, permanent colostomy is required. In low-lying rectal tumors where adequate distal margins and acceptable anal function cannot be secured with LAR, adominoperineal resection (APR) with creation of a colostomy is performed. (NCCN Guidelines*.)

APR is a large operation with en-bloc removal of the rectosigmoid, the rectum, the anus, the mesorectum, and perianal soft tissue. Postoperative wound infections are more common following APR than LAR. Compared to LAR, APR is related to higher incidence of local recurrence and higher mortality as a result of higher incidence of intraoperative tumor perforation and CRM involvement (Marr *et al*. 2005.) Accordingly, alternative techniques with extended tissue removal are now performed in some centers (Holm *et al*. 2007).

2.4.2.3. Adjuvant chemotherapy in rectal cancer

The adjuvant treatment of rectal cancer follows the principles of colon cancer (Schmoll *et al*. 2012), although the data on its efficacy is less established (Dahl *et al.* 2009). Treatment should be started as early as possible, starting from the fourth week up to a maximum of 8-12 weeks after surgery. The length of adjuvant treatment is 5.5-6 months, or 4-4.5 months in the cases where preoperative CRT has been administered. (Schmoll *et al.* 2012). Recently, a meta-analysis including solely rectal cancer patients was conducted to study the efficacy of adjuvant chemotherapy after potentially curative surgery. A significant reduction in the risk of disease recurrence (25%) and death (17%) was seen in patients undergoing adjuvant therapy after surgery compared to those undergoing observation only. The data were not powerful enough to define the effect of chemotherapy in specific disease stages. (Petersen *et al.* 2012.) According to some studies (Collette *et al.* 2007), only patients with tumor downstaging after preoperative CRT could benefit from postoperative adjuvant treatment but this view remains to be concluded.

2.4.3. Surveillance

The aim of the postoperative surveillance is to recognize disease recurrence, premalignant conditions, and adverse effects of the treatments as early as possible (Österlund *et al.* 2012). Wide variation exists in the length and intensity of surveillance programs, although intensive follow-up scheme has been shown to be beneficial in terms of disease outcome (Renehan *et al.* 2002). In addition to anamnesis, routine physical examination, and colonoscopy, CEA is an essential factor in surveillance. Its blood levels increase in 60-80% of patients with recurrent CRC, and increase may be detected before clinical symptoms. CT- and/or PET-imaging are required especially when disease recurrence

is suspected. (Österlund *et al*. 2012.) In the Hospital District of Southwest Finland, radically operated patients are monitored after oncologic treatments at the department of surgery until five years after operation. Thereafter, monitoring may be accomplished in the healthcare center. Colonoscopy is performed at two years after operation, and thereafter every five to ten years.

2.4.4. Treatment of metastatic (stage IV) colorectal cancer

Approximately 20% of CRC patients have synchronous metastases at the time of diagnosis (Howlader *et al*. 2012). In addition, at least another one third with initially curative surgery will develop local and/or distant disease recurrence (Bernstein *et al*. 2012). The most common metastatic sites are liver, lungs, other colonic segments and peritoneum (AJCC Cancer Staging Handbook, 2010). Liver metastases occur in approximately 30% of CRC patients, and account for at least two thirds of deaths from CRC (Kopetz *et al.* 2009). Introduction of oxaliplatin (de Gramont *et al*. 2000), irinotecan (Saltz *et al.* 2000), and biological agents (Hurwitz *et al.* 2004; Cunningham *et al.* 2004; Douillard *et al.* 2010) has enabled increased number of cases to become amenable to hepatic resection, and five-year survival rates of up to 50% have been reported when a complete resection of liver metastases is achieved (Kopetz *et al.* 2009). Chemotherapy and biologic agents also have improved the median OS of patients with unresectable disease from 12 months to 24 months (Glimelius and Cavalli-Björkman, 2012).

Bevacizumab is a monoclonal antiangiogenic antibody that improves response rate (RR), progression-free survival (PFS) time and OS of metastatic CRC as compared to chemotherapy alone (Hurwitz *et al.* 2004). Depending on the type of chemotherapy and line of treatment, PFS is lengthened by approximately two to four months when bevacizumab is combined to chemotherapy (Hurwitz *et al.* 2004; Glimelius and Cavalli-Björkman, 2012). Cetuximab and panitumumab are monoclonal antibodies inhibiting EGFR, and are indicated for the treatment of patients with *KRAS* wild-type (WT) metastatic CRC. Among these patients, treatment with cetuximab or panitumumab improves RR, PFS, and OS when administered together with chemotherapy, or as a monotherapy in chemorefractory disease (Douillard *et al.* 2010; Van Cutsem *et al.* 2011; Glimelius and Cavalli-Björkman, 2012). PFS has been demonstrated to improve by approximately one to two months when compared to chemotherapy or best supportive care alone (Cunningham *et al.* 2004; Douillard *et al*. 2010, Glimelius and Cavalli-Björkman, 2012).

The first-line treatment strategy in metastatic CRC is selected by the basis of disease resectability and aggressiveness, as well as of the symptoms and performance status of a patient (Schmoll *et al.* 2012).

- If the aim is to kill subclinical cancer foci in resectable disease as early and efficiently as possible, neoadjuvant FOLFOX (5-FU, leucovorin and oxaliplatin) combination is often delivered three months before surgery, and the rest of the cycles are given after resection.

- If the aim is to convert a primarily unresectable disease to operable, the most effective treatment is required to allow tumor regression and to kill subclinical disease. Doublet chemotherapeutics with one of the biologic agents are preferred. The biologic agent of choice is cetuximab or panitumumab in *KRAS* WT patients, and bevacizumab in *KRAS*-mutant patients.
- If the aim is purely palliative, treatment should not be too toxic. If rapid tumor regression is demanded because of aggressive disease and severe symptoms, a chemodoublet with or without one of the biologic agents is often a reasonable choice in the first line. If there are no signs of tumor-related symptoms and rapidly progressive disease, an initial single 5-FU-based regimen with or without bevacizumab is a valid option. Watchful waiting may be considered in this group of patients.

Selection of the treatment in second- and subsequent lines is dependent on the choice of the first-line treatment (Schmoll *et al.* 2012). Several issues in the palliative treatment of metastatic CRC remain indecisive, among which are when to start treatment in patients without tumor-related symptoms, and when to stop treatment in the case of a response (Glimelius and Cavalli-Björkman, 2012). The benefit of resecting primary tumor in asymptomatice disease with unresectable metastases also remains an area of continuous discussion (Cirocchi *et al.* 2012). In patients with a symptomatic pelvic mass, palliative RT may be delivered if still possible after previous RT (Schmoll *et al.* 2012). Improved control of liver metastases may be achieved with selective internal radiation therapy (SIRT) in patients following failure on first- and second line treatments with chemotherapy (Cosimelli *et al*. 2012).

2.5. Prognostic factors in colorectal cancer

Prognostic factor is defined as a situation, condition, or a characteristic that can be used to estimate the chance of recovery from a disease or the probability of the disease recurring (NSI Dictionary of Cancer Terms). Prognostic factors are especially important in directing postoperative treatment decision in stage II CRC. Several biomarkers relating to important cellular functions have been evaluated to improve CRC predictability, but none of them is in routine clinical use.

2.5.1. Radicality of resection

Radical resection is a requisite for a permanent cure from CRC. Pathologists play a central role in the assessment of surgical resection specimens. The form used in pathological assessment of CRC specimen at the Department of Pathology, Turku University Hospital, is shown in **Table 6.**

CRM, depicted in **Figure 3,** is the smallest distance between the tumor front and the surgically dissected non-peritonealized surface of a specimen. Thus, it is considered as a

surrogate of quality of surgery especially in rectal cancer, where CRM ≤1mm is a powerful predictor of local recurrence, development of distant metastases, and adverse survival. (Quirke *et al.* 1986; Nagtegaal and Quirke, 2008.) CRM is more often involved after surgery for low rectal cancer compared to that for other locations, originating to challenging anatomical location and higher rate of APR in low-lying tumors (Nagtegaal and Quirke, 2008).

Table 6. The form of pathology report used at Turku University Hospital.

Tumor diameter (mm)
[a] Distance from visceral peritoneum (mm)
[b] Lateral/circumferential margin (mm)
Proximal margin (cm)
Distal margin (cm)
Differentiation grade (I, II, III)
Depth of tumor penetration (pathologic T1, T2, T3, T4a-b)
Vascular/lymphatic invasion (yes/no)
Number of metastatic/examined lymph nodes (metastatic/examined)
[c] Tumor regression grade (<25%, 25-50%, >50%, 100%)

[a] For colon and high rectal carcinoma. [b] For mid and low rectal carcinoma, and colon carcinoma directly infiltrating other peritoneal structures or mesenterial resection margin. [c] For rectal cancer treated with preoperative CRT according to Rödel *et al.* 2005

2.5.2. Pathologic stage

The prognosis of CRC is highly determined by the disease stage that is reported by a pathologist after operation. Pathologic stage is indicated with a prefix "p" (pTNM), or with "yp" (ypTNM) if preoperative CRT has been administered. Invasion through the bowel wall (T3-4) and number of involved lymph nodes (N1-2) are independent high-risk factors for both recurrence and survival (Gunderson *et al.* 2010a; Gunderson *et al.* 2010b). The five-year disease-specific survival rates are rather similar for colon and rectal cancer as shown in **Table 7**. Good quality of pathology is a determining factor for correct staging, as the prognosis of CRC is dependent on the number of examined lymph nodes (Swanson *et al.* 2003). Up to 9% of T1 tumors may present with lymph node metastases (Kobayashi *et al.* 2010), and at least 12 lymph nodes should be identified to accurately rule out nodal metastases (TNM Classification of malignant tumors, 7th edition). The number of identified nodes is often smaller after preoperative RT for rectal cancer than it is in the cases with no treatments prior to surgery (Baxter *et al*. 2005).

2.5.3. Rectal cancer response to preoperative treatment

Tumor regression grade (TRG) is assessed after long-course CRT. It is based on microscopical assessment of the hematoxylin-eosin stained samples to evaluate the amount of residual tumor cells and RT-induced fibrosis (Dworak *et al.* 1997). There are many algoritms which can be used for the evaluation of TRG (Bibeau *et al.* 2011). The most common scales base on five-point Dworak (Dworak *et al.* 1997) or Rödel (Rödel *et al.* 2005) scales, or their three to four point simplified versions (Korkeila *et al*. 2009). TRG ranges from no signs of

regression to pathologic complete response. pCR is seen in 0-27% of cases (Dworak *et al*. 1997; Janjan *et al.* 1999), and associates with improved DFS (Rödel *et al.* 2005).

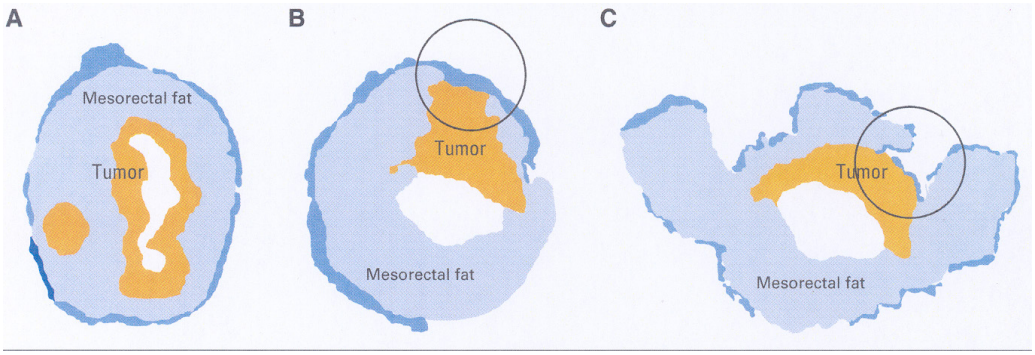

Figure 3. Schematic representation of the circumferential resection margin (CRM). The margin is marked with black ink. (A) Negative CRM. (B) Locally advanced tumor growth, directly into the CRM. (C) Small tumor growing into the CRM as a result of incomplete removal of the surrounding mesorectum. The plane of resection is onto the muscularis propria. Adapted from Nagtegaal ID and Quirke P: J Clin Oncol vol. 10, 2008: p 304. Reprinted with permission. © (2013) American Society of Clinical Oncoloy. All rights reserved.

2.5.4. Histological type and differentiation

Mucinous tumor histological type has been associated with poor prognosis in some, but not all, studies. The prognosis of mucinous tumors has been suggested to depend on the MSI status of a tumor (Verhulst *et al.* 2012). Signet-ring cell carcinoma and serrated adenocarcinoma are prognostically unfavorable, whereas medullary carcinoma is prognostically favorable as compared to conventional adenocarcinoma (Compton, 2002; Mäkinen, 2007; García-Solano *et al.* 2010). Despite there is some lack of standardization concerning tumor grading system, poor differentiation grade is known to be an adverse prognostic factor (Compton, 2002).

Table 7. Relative 5-year survival of colon and rectal cancer according to TNM stage.

Colon cancer	5-year relative survival (%)	Rectal cancer	5-year relative survival (%)
TNM stage, 7th edition		*TNM stage, 7th edition*	
I	97.1	I	93.6
IIA	87.5	IIA	78.7
IIB	79.6	IIB	69.2
IIC	58.4	IIC	53.6
IIIA	68.5-90.7	IIIA	82.7-88.4
IIIB	53.4-81.7	IIIB	46.2-67.7
IIIC	15.7-40.9	IIIC	14.1-53.1
IV	11.7	IV	12.7

Modified based on Gunderson *et al.* 2010a and b; Howlader *et al.* 2012

2.5.5. Invasion parameters

The invasion of tumor cells into lymph or blood vessels is crucial for metastatic process. It is seen in approximately 33% and 23% of non-metastatic CRC cases, and associates with early disease progression and adverse survival especially in the cases where tumor deposits are detected in the veins beyond the muscularis propria (Betge *et al.* 2011). Perineural invasion, characterized as tumor invasion of nervous structures and spread along nerve sheets, is often underreported, although an important adverse prognosticator (Liebig *et al*. 2009).

Tumor border configuration has been shown to have prognostic significance in CRC. Infiltrating growth pattern, with glands invading in a diffuse manner, is associated with poor outcome as compared to tumors with pushing or reasonably well circumscribed border (Jass *et al.* 1987). Similarly, tumor budding, defined as microscopic clusters of undifferentiated cancer cells ahead of the invasive tumor front, indicates poor outcome in CRC (Hörkkö *et al.* 2006). Furthermore, peritumoral deposits of cancer cells away from the invasive tumor front (N1c) have been shown to have prognostic value (AJCC Cancer Staging Handbook, 2010).

2.5.6. Molecular features and serum markers

Strong evidence exists of the prognostic value of MSI status. Patients with MSI-H tumors have higher survival rates than those with MSS tumors (Sargent *et al.* 2010). Peritumoral lymphocytic response and tumor-infiltrating lymphocytes are often present in MSI-H tumors, and associate with favorable prognosis (Hamilton *et al.* 2010).

Many molecular features of a tumor, such as *KRAS*, *BRAF,* and *PI3KCA* mutations, loss of chromosome 18 heterozygosity, and increased expression of thymidylate synthase (TS) and hypoxia-related markers have been suggested as adverse prognostic indicators in CRC (Hamilton *et al*. 2010; Schmoll *et al*. 2012; Korkeila *et al*. 2012). They are not, however, in clinical use. The lack of utility has been proposed to reflect the complexity of CRC (Hamilton *et al.* 2010), and on the other hand, the close adherence of biomarkers with one of the three molecular phenotypes (CIN, MSI, CIMP) showing differing prognoses (Walther *et al.* 2009). Gene expression analyses, such as ColoPrint®, with multiple genes have been shown to be of prognostic value in some studies (Salazar *et al.* 2011), but to date, are not in routine clinical use.

CEA is a serum glycoprotein that is normally produced during embryogenesis. It is often overexpressed in CRC, especially in advanced disease stages. CEA is measured at the time of diagnosis, and its initially high serum concentration associates with adverse prognosis. After radical surgery, serum levels return to normal in four to six weeks. CEA is useful in surveillance, as its serum levels tend to rise during disease progression. (Eche *et al.* 2001.) Carbohydrate antigen 19-9 (CA 19-9) is another serum antigen that has been related to poor prognosis in CRC (Kouri *et al.* 1992), but in contrast to CEA, is not in routine clinical use. Other biochemical factors, such as high alkaline phosphatase, high

lactate dehydrogenase, and high platelet count are poor prognostic factors in metastatic CRC (Schmoll *et al.* 2012).

2.5.7. Other factors

Multidisciplinary teamwork (Palmer *et al*. 2011) and expertise of a practitioner (Hodgson *et al.* 2001) provide benefit in terms of improved tumor control. Tumor obstruction and/or perforation are poor prognostic factors (Schmoll *et al.* 2012). Of the patient-related factors, increasing age, black race, and low socioeconomic status have been associated with worse CRC survival (Howlader *et al*. 2012; Schmoll *et al*. 2012).

2.6. Predictive factors in colorectal cancer

Predictive factor is defined as a condition or finding that can be used to predict whether a cancer will respond to a specific treatment (NSI Dictionary of Cancer Terms). In ideal case, such a factor could help to understand the mechanisms underlying resistance to RT, chemotherapy, and biologic agents, and lead to altered or intensified therapy approaches in non-responding patients. It should be noted that predictive biomarkers may also have prognostic value, and vice versa (Jensen *et al.* 2012). Of the several studied biomarkers, *KRAS* mutation testing is the only one that is routinely utilized in the treatment of colorectal cancer patients.

2.6.1. Predictive markers for response to chemoradiotherapy in rectal cancer

Several radiobiological mechanisms are important for the sensitivity of rectal cancer to preoperative treatment. These mechanisms include the extent of tumor hypoxia, the number of cancer stem cells, the intrinsic radiosensitivity of a tumor, and repopulation capacity of cancer cells during and after RT (Huerta *et al.* 2009; Koukourakis *et al.* 2012). Expression of several tissue-based biomarkers and gene profiles have been studied to better understand the biological factors determining response to CRT. Usually, expression in pre-treatment biopsies is compared to TRG or the rate of pCR, but also alterations in protein levels between pre- and post-irradiation samples have been of interest. Most widely studied genes/biomarkers in this respect are tumor-suppressor genes *TP53* and *p27*, apoptosis-related proteins bcl-2 and bax, proliferation marker Ki-67, receptor tyrosine kinase EGFR, and enzyme TS. Even though some of these markers seem to be promising, none of them has currently enough impact to be used in clinical routine. (Kuremsky *et al.* 2009). Of the serum markers, low pre-treatment concentration of CEA has been associated with good CRT response (Moureau-Zabatto *et al*. 2011).

Lenghtening the interval between CRT and surgery to ≥ 8 weeks has been demonstrated to associate with higher tumor response and improved local control, possibly as a result of cell death continuing over the time after treatment (Kalady *et al.* 2009).

2.6.2. Predictive factors for response to chemotherapeutic agents

To date, no single biomarker is in routine clinical use to predict response to chemotherapeutic agents, although several potential factors have been under extensive studies.

For fluoropyrimidines (5-FU, capecitabine), low expression levels of the enzymes involved in their metabolism, as well as low expression level of TS, which is the target of one of their active metabolites, have been suggested to be predictive of favorable treatment response (Jensen *et al.* 2012). Patients with MSI-H tumors are indicated to receive no benefit from FU-based adjuvant therapy as compared to patients with MSS tumors. The underlying mechanisms might include the high lymphocytic infiltrate characterizing MSI-H tumors together with the deficiency to repair DNA replication errors. (Sargent *et al.* 2012). However, the predictive value of MSI status remains inconclusive, as some other studies indicate reduced risk of recurrence with chemotherapy treatment irrespective of the MSI status (Hutchins *et al*. 2011). Yet others suggest that the negative predictive value of MSI-H status is restricted to those tumors where MSI originates from epigenetic defect, contrary to those with MSI resulting from a germline defect (Sinicrope *et al*. 2011).

As for oxaliplatin, low expression of the protein product of the excision repair cross-complementing gene 1 (*ERCC1*) has been related to a greater benefit of oxaliplatin in some, but not all studies. ERCC1 is involved in the repair of DNA lesions caused by platinum-based chemotherapy agents. (Jensen *et al*. 2012.)

Irinotecan causes breaks in DNA and prevents DNA strand religation leading to subsequent cell death. These effects are executed via inhibition of topoisomerase I. Moderate to high expression of topoisomerase I has been correlated to benefit from irinotecan, but the relation is not widely recognized**.** In addition, UDP-glucuronosyltransferases involved in irinotecan metabolism have gained interest when searching predictive biomarkers for irinotecan. (Jensen *et al.* 2012.)

2.6.3. Predictive factors for response to biological agents

Bevacizumab is a monoclonal antibody against vascular endothelial growth factor (VEGF), an important regulator of angiogenesis. The plasma or tissue levels of VEGF are not predictive of benefit from bevacizumab, although single nucleotide polymorphism in the VEGF system has shown some predictive value (Hansen *et al.* 2012). In contrast to earlier beliefs, there is now some evidence that *KRAS* mutation could relate to inferior benefit from bevacizumab similarly to situation with EGFR-targeted antibodies

(Stintzing *et al.* 2012). During treatment, bevacizumab-induced hypertension has been associated with improved treatment benefit from this antibody (Österlund *et al.* 2011).

Cetuximab and panitumumab are EGFR-targeted monoclonal antibodies with implications in tumor proliferation, apoptosis, angiogenesis and invasion. EGFR is overexpressed in up to 82% of CRCs (Spano and Vignot, 2007), but the expression levels of EGFR protein do not seem to correlate with clinical benefit (Chung *et al.* 2005). Instead, *EGFR* gene copy number (GCN) has been correlated with benefit from EGFR-targeted treatment (Moroni *et al.* 2005; Scartozzi *et al.* 2009; Ålgars *et al*. 2011). Increased activity of other growth factor receptors, namely c-MET, insulin-like growth factor receptor (IGF1R) and human epidermal growth factor receptor 2 (HER-2), have been related to EGFR-targeted treatment response, possibly as a result of bypassing the EGFR signaling pathway (Bardelli and Siena, 2010; Inno *et al.* 2011). Mutation in *KRAS*, a downstream signaling molecule of EGFR, is indicative of lack of benefit from EGFR-targeted therapy (Lièvre *et al*. 2006), and serves as the only predictive marker in clinical use for the treatment of CRC. A small subgroup of patients with *KRAS* mutation in codon 13 has been suggested to be an exception and benefit from EGFR-targeted therapy (De Roock *et al*. 2010b). To date, however, these patients are treated similarly to other patients with *KRAS*- mutated tumors due to lack of firm evidence. In addition to *KRAS*, aberrations in other EGFR downstream effectors such as *BRAF*, *PI3KCA*, and PTEN have been associated with resistance to EGFR-targeted antibodies, and increased expression levels of the EGFR ligands amphiregulin and epiregulin are suggested to indicate benefit from treatment (De Roock *et al.* 2010a; Bardelli and Siena, 2010).

2.7. Biomarkers studied in this thesis

Hanahan and Weinberg suggested that the complexity of cancer may be reduced to ten underlying principles, appointed as the hallmarks of cancer (Hanahan and Weinberg, 2011). Seven biomarkers contributing to these principles were under special focus in this thesis. They are introduced with the respective hallmark in **Figure 4**, and are described in more detail below.

2.7.1. Group IIA secretory phospholipase A2 (IIA PLA2)

Inflammatory component is present in virtually all neoplastic lesions, and associates both with anti- and pro-tumoral actions. Inflammation is evident already at the earliest stages of neoplastic progression, and may contribute to several hallmark capabilities, such as sustaining proliferative signaling, resisting cell death, and promoting invasion and metastasis (Hanahan and Weinberg, 2011).

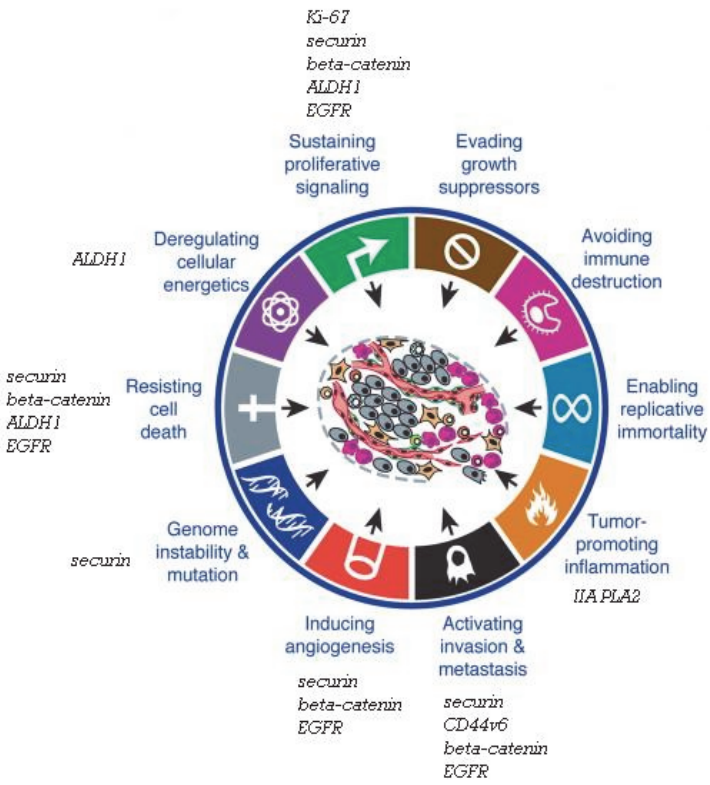

Figure 4. Hallmarks of cancer and the biomarkers studied in this thesis. Adopted and modified from Hanahan D and Weinberg RA: Hallmarks of cancer: the next generation. Cell 2011; 144: p. 668. Molecules mentioned are those discussed in the present thesis.

Phospholipase A2 (PLA2) is a family of proteins that are able to degrade phospholipids from the cell membrane. If arachidonic acid is released from the membrane, it is subsequently converted to eicosanoids and prostaglandins in cyclooxygenase-dependent reactions. Thus, PLA2s play an important role in inflammatory processes. Moreover, cyclooxygenase-2 is overexpressed in colorectal adenomas and carcinomas, and its derivatives are able to signal via the important tumorigenic PI3K- and MAPK-pathways. (Greenhough *et al*. 2009.) The cytosolic isoforms of PLA2s are mainly responsible for arachidonic acid metabolism. Secretory phospholipases A2 (sPLA2) are the largest branch of mammalian PLA2-family, and are often found extracellularly where they are secreted by platelets, inflammatory cells, gastric cells, Paneth cells of the small intestine, and goblet cells of the large intestine. (Fijneman and Cormier, 2008). Their expression is induced by pro-inflammatory cytokines as shown in **Figure 5,** and they have diverse roles in metabolism and innate immunity**.**

Group IIA secretory PLA2 (IIA PLA2) is a calcium-dependent enzyme of 14 kilodaltons (kDa) encoded by a gene located on human chromosome 1p35 (Riggins *et al.* 1995). IIA PLA2 was originally purified from human synovial fluid (Kramer *et al.* 1989), and is known to be expressed in several human tissues (Nevalainen and Haapanen, 1993). The

secretion of IIA PLA2 is induced by several pro-inflammatory cytokines (Fijneman and Cormier, 2008), and dramatically increased serum and tissue concentrations are seen in acute and chronic inflammatory disorders (Rintala *et al.* 1993; Haapamäki *et al.* 1997). Due to cationic nature of IIA PLA2 protein, it is much more efficient in hydrolyzing fatty acids from perturbed, and thus negatively charged, lipid membranes than from healthy cell membranes (Leidy *et al*. 2006). In addition to its direct effects on cell membranes, IIA PLA2 may signal through a cell-surface receptor, inducing activation of pro-inflammatory and pro-tumoral downstream signaling pathways such as MAPK (Fijneman and Cormier, 2008). IIA PLA2 also has bactericidal activity towards gram-positive bacteria (Weinrauch *et al.* 1996; Laine *et al*. 1999), and it is capable of indirectly activate peroxisome proliferator -activated receptors (Fijneman and Cormier, 2008).

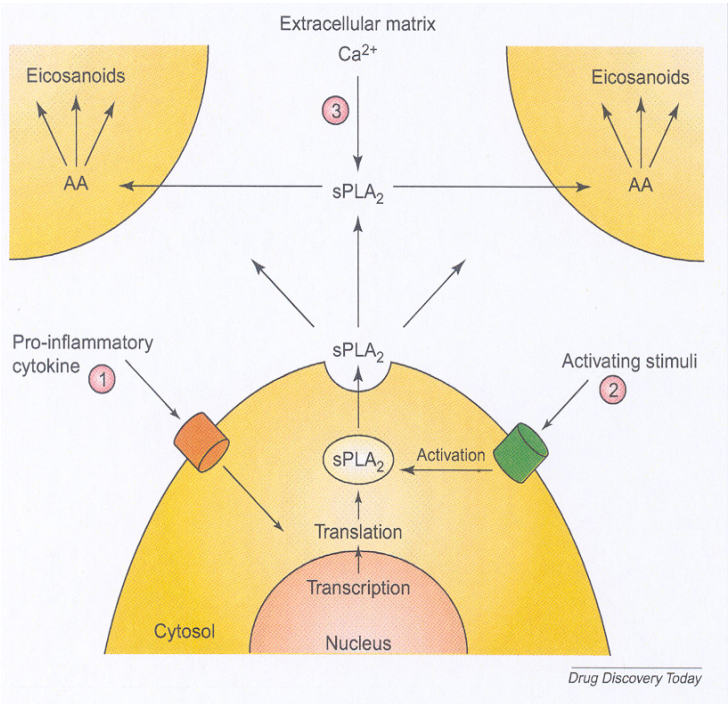

Figure 5. sPLA2 participating in an inflammatory response. (1) Pro-inflammatory cytokines such as tumor necrosis factor α (TNF- α) or interleukin 1β (IL-1 β) induce cellular expression of sPLA2. (2) Activating factors cause release of sPLA2 from secretory granules into the extracellular matrix. (3) In the presence of millimolar concentrations of Ca2+, sPLA2 hydrolyzes membrane-bound phospholipids of neighbouring cells. Released fatty acids, such as arachidonic acid, are further metabolized into eicosanoids, generating an inflammatory response in neighbouring cells. Reprinted from Laye JP and Gill JH: Phospholipase A2 expression in tumours: a target for therapeutic intervention? Drug Discov Today, 2003; vol 8: p.712. Copyright (2013) with permission from Elsevier.

IIA PLA2 has various links to human malignancies such as colorectal, pancreatic and gastric cancer, but it remains controversial whether the role of IIA PLA2 in carcinogenesis actually is pro- or anti-tumoral (Fijneman and Cormier, 2008). On one hand, pro-tumoral effect would be presumed based on its ability to produce arachidonic acid for eicosanoid and prostaglandin synthesis, and on its potential in inducing activation of EGFR-signaling pathways (Hernández *et al.* 2010). Furthermore, IIA PLA2 has been appointed as a transcriptional target of Wnt/β-catenin signaling that is usually mutated

in CRC (Ganesan *et al*. 2008). On the other hand, arachidonic acid is known to induce apoptosis (Cao *et al.* 2000), affording a potential tumor-suppressive role for PLA2s. The presence of IIA PLA2 has also been suggested to protect intestinal epithelium from carcinogenic effects of dietary fatty acids and bacterial products (MacPhee *et al.* 1995). Most importantly, *IIA PLA2* gene corresponds the modifier of Min1 (Mom1) locus in mice, which confers resistance to intestinal tumorigenesis in *APC*-mutated Min-mice (MacPhee *et al.* 1995). Although it is unlike that *IIA PLA2* functions as such a major modifying gene in human FAP-counterparts (Nimmrich *et al.* 1997), dysregulation of IIA PLA2 has been suggested to be a relatively early event in colorectal carcinogenesis (Kennedy *et al.* 1998). Taken together, the association of IIA PLA2 expression and dysregulation with development of CRC remains far from explicit, as both pro- or anti-tumorigenic roles for IIA PLA2 have been suggested in many studies (MacPhee *et al.* 1995; Nimmrich *et al.* 1997; Belinsky *et al.* 2007), and both upregulation (Buhmeida *et al*. 2009) and downregulation (Edhemovic *et al*. 2001) of IIA PLA2 are reported in CRC.

2.7.2. Ki-67 and securin

Persistent cell proliferation is essential for tumor growth. Cancer cells are characterized by unlimited replicative potential due to their lower requirement for growth signals, insensitivity to antigrowth signals, and their ability to escape senescence and subsequent cell death. (Hanahan and Weinberg, 2011). This feature is exploited in treatment modalities, because chemotherapeutics (Jensen *et al.* 2012) and radiotherapy (Pawlik *et al.* 2004; Debucquoy *et al.* 2009) most effectively attack rapidly dividing cells. Irradiation results in either cell-cycle arrest and subsequent DNA repair, or in programmed cell death if the damaged DNA cannot be repaired (Huerta *et al.* 2009). The phases of the cell cycle are shown in **Figure 6.**

2.7.2.1. Ki-67

Ki-67 is a nuclear antigen of 395 kDa encoded by a gene located on chromosome 10q25 (Gerdes *et al.* 1984; Fonatsch *et al.* 1991; Brown and Gatter, 2002). It is a proliferation marker, the expression and localization of which varies throughout the cell cycle phases within the nucleus; levels are low in G1 and S phases, and peak in mitosis. Traditionally, resting phase G_0 cells were deemed as to be devoid of Ki-67 expression, and Ki-67 was known to have no other functional roles than cell proliferation (Gerdes *et al.* 1984; Brown and Gatter, 2002.) Recently, using advanced techniques, some fractions of protein have been detected also in G_0 cells, and Ki-67 has been related in ribosomal DNA transcription or early ribosomal RNA processing events (Bullwinkel *et al.* 2006). Ki-67 expression may be determined from paraffin-embedded tissue specimens using antibodies directed against the protein. In addition to affording additional value for instance in the diagnosis of neuroendocrine tumors (Janson *et al.* 2010), high labeling index of Ki-67 has been shown to have prognostic value in several human malignancies, among others in breast cancer and mesenchymal tumors (Brown and Gatter, 2002).

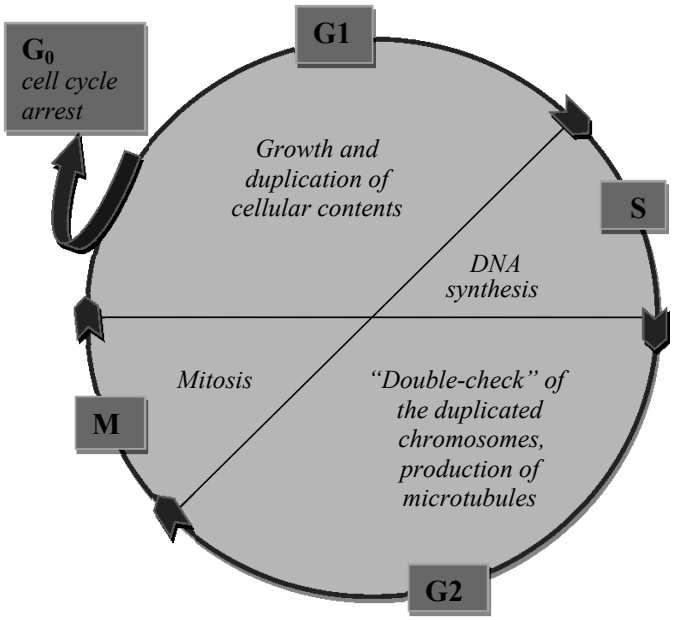

Figure 6. Phases of the cell cycle.

In CRC, the prognostic significance of Ki-67, and thus proliferation rate, remain controversial (Allegra *et al.* 2003; Hilska *et al*. 2005; Bertolini *et al*. 2007) although a continuous increase in proliferative activity is seen along adenoma-carcinoma sequence (Risio *et al.* 1988). Similarly, the predictive value of Ki-67 is confounding in CRC. High pre-irradiation Ki-67 expression has been reported to associate both with beneficial (Willett *et al.* 1995), and adverse (Jakob *et al*. 2008) CRT response, or to be of no significance (Terzi *et al.* 2008).

2.7.2.2. Pituitary tumor transforming factor 1 (PTTG1) and securin

Human pituitary tumor transforming gene 1 (*PTTG1*) is an oncogene located on chromosome 5q33 (Zhang *et al.* 1999). Originally, *PTTG* was isolated from a rat pituitary tumor, and was shown to induce cell transformation *in vitro* and tumorigenesis *in vivo* (Pei and Melmed, 1997). It has both physiological and tumorigenic actions, as it contributes to organ development, cellular proliferation, apoptosis and DNA damage response along with its ability to interact with growth factors. *PTTG1* can also transactivate other genes, thus affecting the amount of several proteins with a potential importance in carcinogenesis (Vlotides *et al.* 2007). Human securin is a 22 kDa protein product *PTTG1* (Domínguez *et al.* 1998) and is involved in the sister chromatid separation at the metaphase-anaphase interface of mitosis (Zou *et al.* 1999; Jallepalli *et al*. 2001). Securin localizes both to cytoplasm and nucleus, and its nuclear translocation is facilitated by a PTTG-binding factor. The role of cytoplasmic protein remains unresolved, while the nuclear one accounts for the biological activity of securin. (Vlotides *et al.* 2007.) Securin expression is cell cycle-dependent with its amount beginning to accumulate at the S-phase and peaking at G_2- and M-phases

(Zou *et al.* 1999). During mitosis, securin is rapidly degraded at the end of metaphase by an anaphase-promoting complex. Accordingly separin, an important regulator of chromosome segragation**,** is released from its tonic inhibition and sister chromatids may equally distribute to diploid daughter cells (Jallepalli *et al.* 2001.) Recently, securin was indicated to have a role also in the regulation of G_1/S-phase transition based on its interaction with the transcription factor Sp1 (Tong *et al.* 2007). Although considered as a critical component in cell cycle regulation, the actual contribution of securin to cell proliferation remains rather inconclusive. Some have reported securin overexpression to cause increased proliferation, while some others reported inhibition of proliferation (Vlotides *et al.* 2007.) Similarly to proliferation, the exact influence of securin on apoptosis remains controversial. Securin is able to interact with p53, an important regulator of cellular apoptosis (Yu *et al.* 2000; Bernal *et al.* 2002), but overexpression of securin has been related both to induced (Yu *et al*. 2000) and decreased rate of apoptosis (Bernal *et al*. 2002). Discrepancies may base on distinct phosphorylation status and subcellular location of securin between the examined cell types and species (Vlotides *et al.* 2007).

In normal human tissues, high levels of *PTTG1* messenger RNA are seen in placenta, adult testis, thymus, and fetal liver, while lower levels are seen in small intestine, colon, brain, placenta, pancreas and lung. Instead, high expression is seen in many carcinoma cell lines and tissues, including CRC (Domínguez *et al.* 1998; Zhang *et al.* 1999.) Several mechanisms are proposed to interconnect *PTTG1* and its protein product securin with cancer development. First, *PTTG1* is able bind to several gene promoters and to upregulate the expression of basic fibroblast growth factor (Zhang *et al.* 1999), vascular endothelial growth factor (McCabe *et al.* 2002), and matrix metalloproteinase 2 (Malik and Kakar, 2006), all of which contribute to increased tumor angiogenesis and invasive potential. Second, dysregulation of securin expression and subsequent defects in chromatid separation contribute to genetic instability, and generation of malignant tumors (Zou *et al*. 1999; Jallepalli *et al.* 2001). Third, securin is involved in DNA damage and repair control by virtue of its ability to interact with and inhibit DNA repair proteins such as p53 and Ku70 (Bernal *et al.* 2002; Kim *et al.* 2007).

As in numerous malignancies, securin is overexpressed also in CRC and it has been correlated with advanced disease stage and adverse prognostic factors (Heaney *et al*. 2000; Wu *et al.* 2008). However, no study has reported its expression to associate with CRC survival (Heaney *et al.* 2000; Talvinen *et al.* 2006; Wu *et al*. 2008) in contrast to many other malignancies (Vlotides *et al.* 2007). As to predictive value, securin expression has been suggested to have an important role in determination of the fate of CRC cells after introduction of DNA-damaging agents (Chen *et al.* 2010), and its expression levels are shown to alter after exposure to chemotherapeutics (Chiu *et al*. 2006) and irradiation (Chen *et al.* 2010). However, there are no previous studies on the predictive value of securin expression on RT response in human rectal cancer.

2.7.3. CD44 variant 6 (CD44v6)

Metastases are the cause of 90% of human cancer deaths. Cancer cells must proceed through a multistep process of local invasion, intravasation to blood and lymphatic vessels, and ultimately, extravasation in order to colonize other organs. Alterations in shape, attachment to other cells and to extracellular matrix (ECM) are seen in cancer cells as early signs of invasive potential. (Hanahan and Weinberg, 2011.) Abnormalities in cellular adhesion in the tumor invasive front, i.e. interface between tumor and surrounding non-tumor tissue, are considered especially important because balance between pro- and anti-tumoral factors in this compartment may be decisive for tumor progression (Suzuki *et al.* 2008).

CD44 is a family of immunologically related cell surface proteoglycans and glycoproteins encoded by a gene located on chromosome 11 (Dalchau *et al.* 1980; Ponta *et al*. 1998). There is significant structural heterogeneity between the CD44 proteins resulting from alternative splicing of ten variant exons, as well as from post-translational modification. The smallest transcript CD44 standard lacks any of the variant exons. It is expressed in most of the tissues of the adult organism, particularly on cells of the hematopoietic system. The larger CD44 variant transcripts present with extracellular domains of variable length due to transcription of selected sequences from variant exons v1-10. In contrast to CD44 standard isoform, the expression of CD44 variants is highly restricted in normal tissues. (Gorham *et al.* 1996; Ponta *et al.* 1998.) CD44 proteins are involved in various physiological and pathophysiological processes including embryogenesis, hematopoiesis, lymphocyte homing, inflammation, and tumor progression (Misra *et al*. 2011). They participate in cell-cell and cell-matrix interactions because their extracellular domain is able to bind among others hyaluronate, collagen and fibronectin (Lesley *et al*. 1993). Some of the CD44 isoforms promote growth factor receptor -mediated signaling by virtue of their ability to bind growth factors, thus affecting cellular proliferation, apoptosis and invasiveness (Bennett *et al*. 1995; Misra *et al*. 2011). The intracellular domain of CD44 is capable of linking cell membrane to cytoskeleton by interacting with actin-binding proteins, thus contributing to cell migration (Tsukita *et al.* 1994). CD44 has also been identified as a potential cancer stem cell (CSC) marker (Dalerba *et al*. 2007), and as such, an important component of chemo- and radiotherapy response (de Jong *et al.* 2010; Croker and Allan, 2012). Through these numerous biological properties, CD44 proteins are considered to possess both growth- and invasiveness promoting functions and tumor-suppressing functions during carcinogenesis (Herrlich *et al.* 2000).

In healthy colon and rectum, CD44 standard may be detected in the lower crypt epithelium, stromal fibroblasts and lymphocytes, whereas expression of CD44 variants is generally absent. In contrast, broad overexpression of CD44 proteins is seen in colorectal adenomas and carcinomas. (Gorham *et al.* 1996.) Pathological conditions promote alternative splicing of CD44 gene, producing variants with enhanced hyaluronate-binding and tumorigenic potential (Sleeman *et al.* 1995; Misra *et al*. 2011). One of these variants

is variant 6 (CD44v6), a membranous and cytoplasmic CD44 protein. The functional role of cytoplasmic protein is rather unknown, but it might contribute to cellular dedifferentiation (Faleiro-Rodrigues and Lopes, 2004). The membranous extracellular domain is responsible for binding of hyaluronate and growth factors, and subsequent activation of intracellular signaling pathways (Misra *et al.* 2011). Overexpression of CD44v6 has been related to increased invasive and metastatic potential (Günthert *et al.* 1991; Wielenga *et al.* 1993; Bendardaf *et al*. 2005), as well as to poor disease outcome (Ropponen *et al.* 1998) in CRC. On the other hand, others have reported favorable outcome (Zlobec *et al.* 2009) and response to chemotherapy (Bendardaf *et al.* 2004) in patients with CD44v6 expressing tumors. Apparently, expression does not remain stable along CRC progression, as it has been shown to both increase (Wielenga *et al*. 1993) and to decrease (Bendardaf *et al.* 2006) during metastatic process.

2.7.4. β-catenin and aldehyde dehydrogenase 1 (ALDH1)

Stem cells are scarce in normal colorectal mucosa. They are characterized by their ability to regulate the balance between self-renewal and cellular differentiation. Cancer stem cells (CSC) are suggested to evolve from and share similarities to normal stem cells (Reya *et al.* 2001), but they also are capable of initiating and sustaining malignant growth (Reya *et al*. 2001) that are considered as hallmarks of cancer (Hanahan and Weinberg, 2011). CSCs self-renew and differentiate in an aberrant, unregulated manner, resulting in stem cell overpopulation and a bulk tumor population (Reya *et al.* 2001; Huang *et al*. 2009) as shown in **Figure 7.** Therapeutic approaches that are unable to eradicate CSCs may be unsuccessful, because even a small subset of CSCs is suggested to allow tumor regrowth (Reya *et al.* 2001). Several cell surface markers have been proposed as CRC stem cell markers due to their aberrant expression in CRC.

2.7.4.1. β-catenin

Activation of the Wnt/β-catenin signaling pathway is considered crucial for the maintenance of both normal and cancer stem cells in the gut epithelium (Reya *et al.* 2001). β-catenin is a protein encoded by a gene located on chromosome 3p21 and is found in the cell membrane, cytoplasm and nucleus (Kraus *et al.* 1994). In the membrane, it binds among others E-cadherin, thus contributing to the formation of adherens junctions that are essential for cellular adhesion. The role of cytoplasmic and nuclear β-catenin is the transduction of the Wnt-signals from the cell surface to the nucleus. In the absence of Wnt-signaling, cytoplasmic β-catenin is rapidly degraded by a multiprotein destruction complex including APC protein. In the presence of Wnt-signaling, accumulation of cytoplasmic β-catenin leads to its nuclear translocation, and binding to transcription factors modulating a broad range of Wnt-target genes. These genes contribute to several physiological and pathophysiological processes including development, CSC signaling, cell proliferation and differentiation, apoptosis, angiogenesis and epithelial-mesenchymal transition (EMT). (Le *et al.* 2008; Yao *et al.* 2011.)

In a normal colonic epithelium, strong nuclear β-catenin staining is detected only at the basal positions of the crypts, decreasing towards the more differentiated cells in the surface epithelium. Instead, cells harboring mutations in *APC* or β-catenin become independent of the physiological signals controlling Wnt-signaling activity, and continue to proliferate and express Wnt-target genes also upper in the surface epithelium. (van de Wetering *et al.* 2002.) This constitutive activation of Wnt-signaling is commonly seen in CRC, where *APC* gene is often mutated in early phases of carcinogenesis as previously described (chapter *2.2.1*). Several biologic inhibitors and small-molecule compounds have thus been studied in order to inhibit Wnt/β-catenin signaling, but their clinical use has so far been hindered by their adverse effects on normal stem cells and tissues (Yao *et al.* 2011).

In CRC, β-catenin has not been proved to be a good indicator of CSCs or disease outcome. Instead, the assessment of intratumoral distribution of nuclear β-catenin may significantly improve the prognostic value of this protein (Horst *et al.* 2009) as β-catenin is not uniformly distributed within a tumor. Cells in the central tumor parts tend to retain their membranous β-catenin expression, whereas nuclear expression is predominant in cells localized at the invasive front, probably reflecting the importance of the surrounding stroma in regulating the Wnt-signaling. (Brabletz *et al*. 1998; Le *et al*. 2008.)

2.7.4.2. Aldehyde dehydrogenase 1 (ALDH1)

Aldehyde dehydrogenase 1 (ALDH1) is a protein encoded by a gene located on chromosome 9q21 (Hsu *et al.* 1989), and it belongs to a group of ALDH-enzymes catalyzing the oxidation of aldehydes to carboxylic acids (Marchitti *et al.* 2008). By virtue of its detoxifying function, ALDH1 plays a key role in the cellular defense against oxidative stress, as well as in resistance to DNA-damaging agents (Marchitti *et al.* 2008; Dylla *et al.* 2008; Chen *et al.* 2009). The main ALDH1 isoenzyme, ALDH1A1, is a cytoplasmic enzyme found in many healthy human tissues including testis, brain, eye lens, liver, kidney, lung and retina (Marchitti *et al.* 2008), where it is able to modulate neurotransmission, cell proliferation, differentiation, and apoptosis by forming retinoic acid (Yoshida *et al.* 1992; Marchitti *et al.* 2008). ALDH1 has been suggested to mark both normal and cancer stem cells in various tissues, including large intestine (Cheung *et al*. 2007; Ginestier *et al.* 2007; Huang *et al*. 2009). Increased ALDH1 expression is seen in several human malignancies (Deng *et al.* 2010), where it has been associated with increased invasive and metastatic potential (Wang *et al.* 2012; Wakamatsu *et al.* 2012), and poor clinical outcome (Ginestier *et al.* 2007; Jiang *et al*. 2009; Wang *et al*. 2012). As for gastrointestinal malignancies, increased expression of ALDH1 protein is seen in esophageal (Wang *et al.* 2012), gastric (Wakamatsu *et al*. 2012), colorectal (Hessman *et al*. 2012), and pancreatic cancer (Kahlert *et al*. 2011).

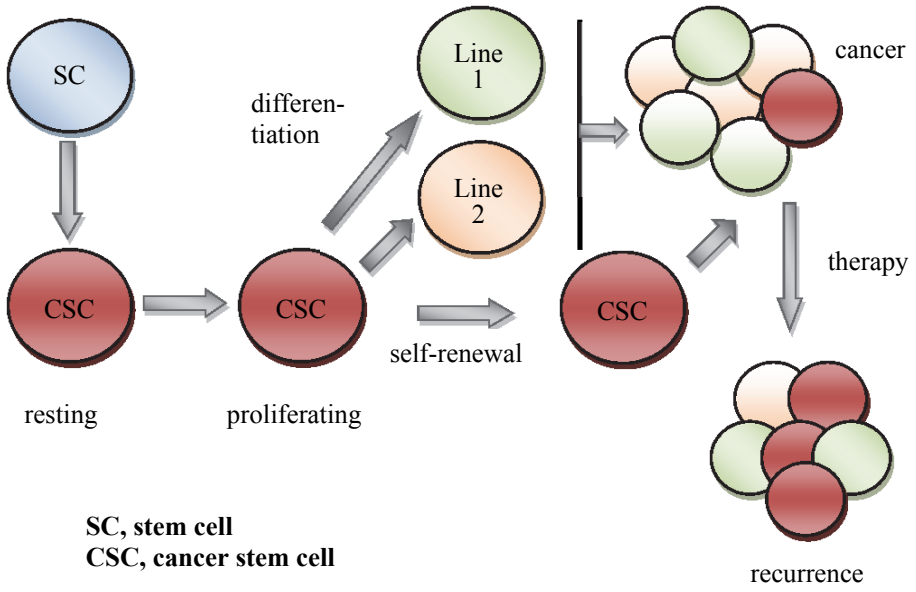

Figure 7. The potential roles of cancer stem cells (CSCs) in the development of cancer. CSCs may arise from normal stem cells through transformation. They have the ability to differentiate and self-renew, enabling tumor formation. CSCs are considered to be more resistant to cancer therapeutics, and they may be overpresented in recurrent disease. Based on Reya *et al*. 2001; and Seufferlein *et al.* 2009.

Progressive colonic stem cell overpopulation during tumorigenesis has been suggested to drive CRC development. Indeed, ALDH1 expression increases during colorectal carcinogenesis from normal epithelium to adenoma and carcinoma (Huang *et al.* 2009). On the other hand, expression has been shown to be decreased in metastatic CRC compared to primary CRC (Hessman *et al*. 2012). The prognostic value of ALDH1 in CRC is rather confusing; while others have shown CRC outcome to be independent of ALDH1 expression (Lugli *et al.* 2010; Hessman *et al.* 2012), others have reported that two prognostically diverging groups may be defined based on their ALDH1 expression pattern (Vogler *et al.* 2012). Moreover, it has recently been suggested that instead of cytoplasmic ALDH1A1, mitochrondrial ALDH1B1 might be the predominant ALDH1 isoform in CRC (Chen *et al.* 2011), and that ALDH1A1 could in rare cases be detected also in the nucleus, potentially relating to poor clinical outcome (Kahlert *et al.* 2012).

Similarly to other malignancies, chemotherapy resistant colon cancer cells are considered to be enriched for CSCs (Dylla *et al.* 2008; Oh *et al*. 2011). FOLFOX is a standard chemotherapy regimen utilized in the treatment of CRC, and FOLFOX-resistant colon cancer cells were found to have markedly higher ALDH1 activity than their corresponding parental cells (Oh *et al.* 2011). With regard to RT, ALDH1 expression has been associated with radioresistance in some malignancies (Chen *et al.* 2009; Croker and Allan, 2012), but its expression has not been studied in relation to CRT response in human rectal cancer.

2.7.5. Epidermal growth factor receptor (EGFR)

Growth factors and their receptors contribute to several hallmarks of cancer via their downstream signaling pathways (Hanahan and Weinberg, 2011). EGFR is a 170 kDa cell-surface receptor tyrosine kinase (Carpenter *et al.* 1978) encoded by a gene localized to chromosome 7p12-13 (Davies *et al.* 1980). It serves as a receptor for the members of epidermal growth factor-family of extracellular ligands, and is closely related to other members of the ErbB receptor-family, namely HER2, Her 3 and Her 4. Upon binding its ligand, dynamic conformational changes occur in both extra- and intracellular domains of the EGFR receptor, resulting in the phosphorylation of tyrosine residues in the carboxyl-terminal regulatory domain, and subsequent binding of downstream molecules. The main downstream signaling pathways regulated by EGFR are the PI3K-pathway, Ras-MAPK-pathway, Janus kinase (JAK)-Signal transducer and activator of transcription (STAT)-pathway, and phospholipase c-γ pathway. (Cohen *et al.* 2003.) Regulated EGFR-signaling is implicated in the maintenance of cell proliferation, differentiation, and other normal cellular processes. Instead, unregulated and aberrant activation contributes to tumor development and progression subsequent to increased cell proliferation, invasion and angiogenesis, as well as to evasion of apoptosis (Spano and Vignot, 2007.)

Activation of the EGFR signaling pathway is considered as relatively early event in colorectal carcinogenesis. Although overexpression of EGFR has been related to advanced disease stage (Karameris *et al.* 1993) and metastatic potential (Radinsky *et al.* 1995), the prognostic value of EGFR overexpression remains inconclusive in CRC (Spano and Vignot, 2007). Neither is it valuable in predicting RT response of rectal cancer due to controversial data (Giralt *et al.* 2005; Zlobec *et al.* 2008). Activating mutations in the *EGFR* gene are rare in CRC (Metzger *et al.* 2011), and alterations in *KRAS* and other downstream signaling molecules together with crosstalk among different growth factor receptors are considered to be more important factors for unregulated EGFR signaling (Lièvre *et al.* 2006; Bardelli and Siena, 2010; De Roock *et al.* 2010a).

EGFR-targeted monoclonal antibodies cetuximab and panitumumab are utilized in the treatment of metastatic CRC. Although several molecules have been proposed as predictive factors for response to these therapies (*chapter 2.6.3*), *KRAS* mutation analysis is the only one in clinical use (Lièvre *et al.* 2006). However, 30% of the non-responsive population do not present with any of the proposed mutations (*KRAS, BRAF, PI3KCA,* loss of PTEN) and consequently, additional predictive factors are required (Bardelli and Siena, 2010). EGFR IHC has been disappointing with this respect. EGFR expression rates are highly variable in the literature (Spano and Vignot, 2007), and also EGFR-negative tumors have shown to benefit from treatment (Chung *et al*. 2005). Consequently, other techniques such as chromogenic (CISH), fluorescence (FISH) and silver-enhanced *in situ* hybridization (SISH) have been introduced to improve the predictability of response to EGFR-targeted agents. Indeed, increased *EGFR* GCN has been associated with improved treatment response in several studies, but with diverse cut-off values and techniques (Moroni *et al*. 2005; Scartozzi *et al*. 2009; Ålgars *et al*. 2011). SISH is a fully

automated chromogenic assay using metallic silver to detect single and amplified gene and chromosome copies (Dietel *et al*. 2007). It is in routine clinical use for determining *HER2* status in breast and gastric cancer, and was recently reported to be valuable in determining *EGFR* GCN and its association with EGFR-targeted therapy response in metastatic CRC (Ålgars *et al.* 2011).

Intratumor heterogeneity in *EGFR* gene expression has been shown among others in colorectal cancer (Yang *et al.* 2012) and glioblastoma (Snuderl *et al.* 2011). It is defined as the coexistence of cancer cell clones with distinct genetic or gene expression profiles and distinct biologic properties within a same tumor (Marusyk *et al.* 2012). Some of the difficulties encountered in validating predictive biomarkers for targeted therapies have been related to this heterogeneity, because underestimation of the tumor mutational burden may occur when the analyses base solely on single tumor-biopsy specimens and scoring of the dominant phenotype (Snuderl *et al.* 2011; Gerlinger *et al*. 2012; Misale *et al.* 2012).

3. AIMS OF THE STUDY

Treatment of CRC is challenging for a patient and physician because of troublesome symptoms, lack of prognostic (*chapter 2.5*) and predictive (*chapter 2.6*) biomarkers, and disease heterogeneity. A substantial need for prognostic and predictive biomarkers exists in order to distinguish patients with different outcomes and responses to oncologic treatments. To date, no tissue-based prognostic or predictive molecular markers exist in clinical use for stage I-III disease, and *KRAS* mutation test is the only one for metastasized disease.

Malignant cells exploit several mechanisms appointed as hallmarks of cancer (Hanahan and Weinberg, 2011) to enlarge tumor mass and to acquire metastatic potential. The aim of this study was to offer novel information about the prognostic and predictive value of selected biomarkers contributing to these hallmarks, using lesions presenting different phases of colorectal carcinogenesis.

The specific aims of this study were:

1) To study the localization of inflammatory mediator IIA PLA2 expression at messenger ribonucleid acid (mRNA) and protein level in colorectal tumors, and to detect whether the expression shows different pattern between benign, premalignant and malignant colorectal lesions.

2) To investigate securin protein expression and its relation to proliferation marker Ki-67 in rectal cancer, to detect the potential impact of (C)RT on securin and Ki-67 expression, and to examine the prognostic and predictive value of the two proteins.

3) To study the expression of CD44v6 at protein level, to evaluate potential differences in its intratumoral expression pattern, and to analyze the prognostic value of CD44v6 in rectal cancer.

4) To study the expression of cancer stem cell marker ALDH1 and its relation to β-catenin in rectal cancer, to analyze the influence of preoperative (C)RT on ALDH1 expression, and to examine the prognostic and predictive value of ALDH1.

5) To validate the predictive value of *EGFR* GCN on EGFR-targeted treatment response in metastatic CRC and CRC cell lines, and to study whether intratumoral heterogeneity significantly affects the results of GCN analyses.

4. PATIENTS AND METHODS

4.1. Patients, tumors and cell lines

The studies were retrospective in nature. The number of biopsy and operative samples in each study is shown in **Figure 8.** A more detail description of the patients and tumors are presented in original publications **I-V**. The use of archival tissue material was approved by the National Supervisory Authority for Welfare and Health (permissions # Dnro 1709/32/300/02, May 13th 2002, and # Dnro 4423/32/300/02, October 15th 2002).

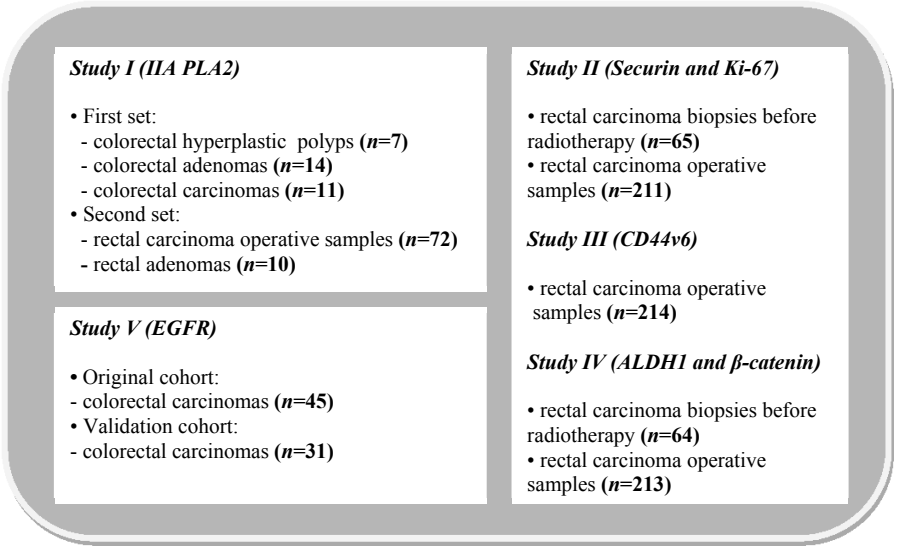

Figure 8. The number of tumors in the studies I-V.

4.1.1. Hyperplastic polyps, adenomas, and carcinomas (I)

As shown in **Figure 8,** the material comprised two distinct sets of tumors. The 72 rectal carcinomas in the second set presented the surgery-only group of studies II-IV. In ten cases, an adenoma was found in the same sample with a carcinoma.

4.1.2. Rectal carcinomas (II-IV)

The material consisted of 227 stage I-III rectal carcinomas situated in middle or lower third of the rectum. Patients were treated at Turku University Hospital between 2000 and 2009. For the studies II and IV, 65 and 64 biopsy-surgical sample pairs were available, respectively. The biopsies included those from preoperatively irradiated patients (short- or long-course). To ensure biologically and therapeutically homogenous study population, following cases were excluded: high rectal carcinomas, superficial tumors treated with local excision, and carcinomas with synchronous metastases.

Table 8a. TNM classification of colon and rectum carcinoma, 6th edition.

T – Primary Tumor	N – Regional Lymph Nodes
TX Primary tumor cannot be assessed **T0** No evidence of primary tumor **Tis**[1] Carcinoma in situ: intraepithelial or invasion of lamina propria **T1** Tumor invades submucosa **T2** Tumor invades muscularis propria **T3** Tumor invades subserosa or into non-peritonealized pericolic or perirectal tissues **T4** Tumor directly invades other organs or structures[2,3] and/or perforates visceral peritoneum	**NX** Regional lymph nodes cannot be assessed **N0** No regional lymph node metastasis **N1** Metastasis in 1-3 regional lymph nodes **N2** Metastasis in 4 or more regional lymph nodes
Notes: **1**. Tis includes cancer cells confined within the glandular basement membrane (intraepithelial) or mucosal lamina propria (intramucosal) with no extension through the muscularis mucosae into the submucosa. **2**. Direct invasion in T4 includes invasion of other segments of the colorectum by way of the serosa, e.g. invasion of sigmoid colon by carcinoma of the caecum. **3**. Tumor that is adherent to other organs or structures, macroscopically, is classified T4. However, if no tumor is present in the adhesion, microscopically, the classification should be pT3.	**Note:** A tumor nodule in the pericolic/perirectal adipose tissue without histological evidence of residual lymph node in the nodule is classified in the pN category as a regional lymph node metastasis if the nodule has the form and smooth contour of a lymph node. If the nodule has an irregular contour, it should be classified in the T category and also coded as V1 (microscopic venous invasion) or V2, if it was grossly evident, because there is a strong likelihood that it represents venous invasion.
	M – Distant Metastasis
	MX Distant metastasis cannot be assessed **M0** No distant metastasis **M1** Distant metastasis

International Union Against Cancer (UICC). Sobin L, Wittekind C (eds). Colon and Rectum *in* TNM classification of malignant tumours, 6th edition, 2002, Wiley-Liss, New York, NY

Table 8b. Stage grouping of colon and rectum carcinoma.

Stage 0	Tis	N0	M0
Stage I	T1, T2	N0	M0
Stage IIA	T3	N0	M0
Stage IIB	T4	N0	M0
Stage IIIA	T1, T2	N1	M0
Stage IIIB	T3, T4	N1	M0
Stage IIIC	Any T	N2	M0
Stage IV	Any T	Any N	M1

International Union Against Cancer (UICC). Sobin L, Wittekind C (eds). Colon and Rectum *in* TNM classification of malignant tumours, 6th edition, 2002, Wiley-Liss, New York, NY

Sixth edition of TNM classification of malignant tumors (TNM Classification of Malignant Tumors, 6th edition, **Table 8a and 8b**) was utilized for pre- and postoperative tumor staging, as it was the contemporary edition at the time of diagnosis. Preoperative

staging included digital rectal examination, CT or MRI of the rectum (solely MRI since 2006), CT of the abdomen, and X-ray or CT of the chest. Patients were studied as three groups according to their preoperative treatment, which followed the clinical guidelines of rectal cancer treatment (*chapter 2.4.2.1*). The total number of patients in each group was as follows: 96 in the short-course RT group, 55 in the long-course (C)RT group, and 76 in the surgery-only group, respectively. Altogether 46 patients (84%) in the long-course (C)RT group received concomitant chemotherapy (5-FU or capecitabine) with radiotherapy. The number of patients in the studies, as well as distribution of disease stages and outcomes, are summarized in **Table 9.** The reasons for missing cases were: unavailability of a sample, pCR after preoperative CRT, and finished/insufficient amount of cancerous tissue in a sample.

The type of surgery among the 227 patients was as follows: LAR for 127 (56%), APR for 96 (42%), and some other technique for four (2%) patients. Surgery was macroscopically radical in 98% of the cases. Pathologic features of the resected specimens (all 227 cases) are presented in **Table 10.** Median number of examined lymph nodes was 12. In 55% of cases, at least 12 lymph nodes were harvested by a pathologist.

Postoperative adjuvant chemotherapy was administered following mainly the common clinical guidelines for stage III and high-risk stage II patients (Schmoll *et al.* 2012). Altogether 111 (49%) patients received postoperative treatment with following regimens: 5-FU in 11 (10%), capecitabine in 66 (60%), combination therapy with oxaliplatin in 21 (19%), and postoperative RT with 5-FU or capecitabine in 13 (11%) cases.

Table 9. The number and characteristics of the patients and tumors in the studies II-IV*.

Variable	Securin, *n* (II)	CD44v6, *n* (III)	ALDH1, *n* (IV)
Number of patients	211	214	213
▪ short-course RT group	87	90	89
▪ long-course (C)RT group	54	53	50
▪ surgery-only group	70	71	74
Sex			
▪ male	119	123	119
▪ female	92	91	94
Mean age	68.1	68.1	68.3
Postoperative stage			
▪ stage I	56	56	56
▪ stage II	68	71	71
▪ stage III	84	84	83
▪ no tumor left	3	3	3
Disease-specific outcome			
▪ alive, no recurrence	137	128	113
▪ alive with recurrence	17	18	14
▪ died of disease	36	41	53
▪ died of other causes	21	27	33

*more detail descriptions are found in the original publications.

Table 10. Pathologic features of the 227 rectal carcinomas.

Variable	*n* (%)	Variable	*n* (%)
[a] *Extent of tumor*		*Circumferential margin*	
(y)pT0	4 (2)	0 mm	19 (8)
(y)pT1	12 (5)	0 < crm < 1	14 (6)
(y)pT2	71 (31)	1-2 mm	10 (4)
(y)pT3	121 (53)	> 2 mm	128 (57)
(y)pT4	19 (9)	Not reported	56 (25)
[a]*Regional lymph node metast.*		[b] *Differentiation grade*	
(y)pN0	137 (60)	Well differentiated	34 (15)
(y)pN1	58 (26)	Moderately differentiated	144 (63)
(y)pN2	29 (13)	Poorly differentiated	40 (18)
Not reported	3 (1)	Not reported	9 (4)
Vascular invasion		*Histology*	
Present	48 (21)	Adenocarcinoma	214 (94)
Not present	119 (52)	Mucinous adenocarcinoma	9 (4)
Not reported	60 (27)	Not reported	4 (2)

[a] T and N according to TNM classification of malignant tumours, 6th edition.
[b] Differentiation grade according to WHO Classification of Tumors of the Digestive System, 4th edition. *Abbreviations:* the prefix "p" indicates pathologic, i.e. determination of T and N by a pathologist from the operative sample. The prefix "yp" indicates pathologic determination when preoperative multimodality treatment has been administered.

4.1.3. Metastatic colorectal carcinomas (V)

The material consisted of two individual sets of colorectal carcinomas from patients treated for metastatic disease at Turku and at Helsinki University Hospitals. The first set ("original cohort") included 45 patients of a previous study by our group (Ålgars *et al.* 2011). The second set with 31 patients was used as a validation material for that study ("validation cohort"). Initially, most of the tumors presented with metastasis to regional lymph nodes (stage III, 28%) or to distant sites (stage IV, 55%) at the time of CRC diagnosis. Primary tumor was situated in the colon in 54 cases (71%), and in the rectum in 22 (29%) cases. All tumors were *KRAS* WT. Patients were treated with EGFR-targeted monoclonal antibody (cetuximab or panitumumab) for their metastatic disease. Altogether 54 patients (71%) received EGFR-targeted therapy in third or subsequent lines, enabling examination of *EGFR* GCN in a chemorefractory group of patients. Treatment regimens and lines for the validation and chemorefractory patient cohorts are presented in more detail in the respective manuscript **V**.

4.1.4. Colorectal cancer cell lines (V)

A search of the Sanger Center cancer cell line database (http://www.sanger.ac.uk/cgi-bin/genetics/CGP/cghviewer /CghHome.cgi) was performed to detect the incidence of *EGFR* GCN increase in CRC cell lines. Four human CRC cells lines with different *EGFR* GCN were purchased for the study V purpose, and are presented in **Table 11**

together with the growth media used**.** All growth media were supplemented with 10% Fetal Bovine Serum (FBS), 2nM glutamine and 1% penicillin/streptomycin.

Table 11. Cell lines used in the study V.

Cell line	Provider	*KRAS*	*EGFR* GCN	Growth media used
C2BBe1	ATCC, Manassas, CA, USA	WT	4	DMEM
NCI-H747		mutated	>4	RPMI-1640
SK-CO1		mutated	>4	EMEM
CW-2	RIKEN Bioresource Center, Tsukuba, Japan	WT	2	RPMI

Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium; EMEM, Eagle's Minimal Essential; RPMI, Roswell Park Memorial Institute; WT, wild type.

4.2. Immunohistochemical stainings (I-V)

4.2.1. Antibodies and procedures

For study I-IV purposes, formalin-fixed paraffin-embedded blocks were cut into 5 µm sections, and for study V purpose to 3 µm sections. Antigen retrieval was performed by heating in microwave oven in 10 mmol/L sodium citrate, pH6, two times for 7 minutes. For study IV, pH9 was used. Endogenous peroxidase activity was blocked with incubating the slides in 0.3% hydrogen peroxide in Tris-buffered saline (TBS). Thereafter, sections were subjected to IHC staining with antibodies that are described in more detail in **Table 12.** The staining procedures are presented in the respective publications.

4.2.2. Evaluation of immunostainings

Evaluation of immunostaining for each antibody is summarized in **Table 13**, and more detail descriptions are provided in the original publications. For IIA PLA2, CD44v6, ALDH1 and β-catenin, the intratumoral localization of positive immunostaining was also assessed. For IIA PLA2, the number of cases with immunopositive cancer cells in the tumor invasive front was reported. For β-catenin, the intratumoral distribution of positive nuclear staining was analyzed as described in the respective publication **(IV).** For CD44v6 and ALDH1, the localization was considered as follows: positive immunostaining present

- equally in the invasive front and central tumor parts,
- mainly in the tumor invasive front, or
- mainly in the central tumor parts.

Moreover, tumor growth pattern was analyzed using Pan-cytokeratin staining in study III. Using Jass' classification (Jass *et al.* 1986), tumor was appointed as "expanding" when the invasive border was pushing or reasonably well circumscribed, and "infiltrating" when the tumor invaded in a diffuse manner with wide penetration into adjacent tissues.

4.3. In situ (ISH) and silver-enhanced *in situ* hybridization (SISH) (I, V)

To analyze the location of IIA PLA2 protein synthesis at mRNA level in colorectal tumors, *in situ* hybridization (ISH) was performed in formalin-fixed, paraffin-embedded tissue samples of four hyperplastic polyps, 12 adenomas, and nine carcinomas. Human group II PLA2 anti-sense (test) and sense (control) single-stranded RNA riboprobes were used as described in the publication **(I).** Similarly to IIA PLA2 IHC, cases with over one percent of positive IIA PLA2 ISH were considered as positive.

Table 12. Antibodies used in the studies.

Study	Antibody	Dilution	Provider	Cat #
I	Polyclonal rabbit anti-human IIA PLA2 IgG	[a]	[b]	
	Mouse monoclonal anti-human IIA PLA2	1:3000	Cayman Chemicals, Ann Arbor, USA	160500
II	Mouse monoclonal anti-human securin	1:100	Abcam, Cambridge, UK	ab3305
	Mouse monoclonal anti-human Ki-67	1:100	Dako, Glostrup, Denmark	M7240
III	Mouse monoclonal anti-human CD44var (v6)	1:1000	Bender MedSystems, Vienna, Austria	BMS 125
	Mouse monoclonal anti-human cytokeratin (Pan)	1:50	Invitrogen Corporation, Camarillo, USA	18-0132
IV	Mouse monoclonal anti-human ALDH1	1:1000	BD Transdusction Laboratories, San Jose, USA	61195
	Mouse monoclonal anti-human β-catenin	1:3000	Invitrogen Corporation, Camarillo, USA	13-8400
V	Rabbit monoclonal anti-human EGFR (5B7)	Ready-to-use	Ventana Medical Systems/ Roche Diagnostics, Tucson, USA	790-4347

[a] IgG fraction (1.04 μg/ml) of polyclonal rabbit anti-human group II PLA2 antiserum was used in a dilution of 1:3000; [b] Inhouse antibody by Nevalainen *et al.* 1993. Cat # indicates catalog number.

For *EGFR* GCN analysis, silver-enhanced *in situ* hybridization (SISH) was performed in 5-μm sections with EGFR DNA probe (Ventana/Roche). SISH was performed with the BenchMark XT using *ultra*VIEW SISH Detection Kit (Ventana/Roche). *EGFR* GCN was evaluated using two approaches. First, with a guidance of the areas showing the highest IHC reactivity, 40 tumor cells with the highest GCN were selected in every slide

(*n*=76). Mean GCN was thereafter counted from these 40 cells. Second, to evaluate *EGFR* GCN heterogeneity in CRC, five areas from the slides of the validation set (*n*=31) were arbitrarily chosen by two observers independently of EGFR IHC. *EGFR* GCN from 20 tumor cells was thereafter counted in each of these five areas, and their mean value was compared to the results obtained from the first analysis. Based on a previous study of our group (Ålgars *et al.* 2011), GCN four was utilized as a cut-off value to distinguish patients with increased (≥4) *EGFR* GCN.

Table 13. Evaluation of immunostaining in the studies.

Study	Cut-off for positive/ high staining	Analyzed staining	Grading of staining intensity	κ-value between observers
I (IIA PLA2)	1% [a]	mainly cytoplasmic	from 0 (negative) to 3 (strong) [b]	N/A
II (securin)		nuclear	from 1 (weak) to 3 (strong)	
short-course RT	30%			
long-course (C)RT	22%			
surgery only	34%			0.83-0.91
II (Ki-67)		nuclear	N/A	
short-course RT	51%			
long-course (C)RT	58%			
surgery only	70%			
III (CD44v6)	20%	cytoplasmic, membranous	from 0 (negative) to 3 (strong)	0.70-90
IV (ALDH1)	3%	cytoplasmic	from 0 (negative) to 3 (strong)	0.90
IV (β-catenin)	N/A	nuclear	N/A	
V (EGFR)	10%	cytoplasmic, membranous	from 0 (negative) to 3 (strong)	N/A

[a] For cancer cells; [b] for peritumoral mucosa; N/A, not analyzed

4.4. KRAS mutation analysis (V)

The most common *KRAS* point mutations within codons 12 and 13 were analyzed from formalin-fixed, paraffin-embedded tissue material including at least 30% of CRC cells. Testing was performed using the DxS K-RAS mutation kit (DxS Ltd, Manchester, UK).

4.5. Evaluation of tumor response to (chemo)radiotherapy (II-IV)

Three-point modification of Dworak (Dworak *et al.* 1997) and Rödel (Rödel *et al.* 2005) scales was used to analyze tumor response to long-course preoperative (C)RT. Tumor

regression grade (TRG) was analyzed in hematoxylin- and eosin-stained sections by a pathologist (JS), and was designated as poor, moderate, or excellent. In the two latter cases, altogether two to eight slides were evaluated to exclude too optimistic determination of the response. In poorly responding tumors, a minimal or no regression was seen after (C)RT. Instead, a considerable tumor mass was seen at the time of operation. Moderate response was defined as a few tumor cell groups or glands present in the primary tumor after preoperative treatment. In the case of excellent response, very few or no tumor cells were left after preoperative (C)RT.

4.6. Evaluation of response to EGFR-targeted therapy (V)

The response to cetuximab and panitumumab was evaluated with CT or MRI following the Response Evaluation Criteria in Solid Tumors (Eisenhauer *et al.* 2009). Patients with complete response, partial response, or stable disease were considered as having received clinical benefit from EGFR-targeted treatment. The study was conducted in accordance with the Declaration of Helsinki.

4.7. Western blotting and cytotoxicity assays (V)

Among the cell lines, *EGFR* GCN was confirmed with SISH, whereas the amount of protein was analyzed with Western blotting, as described in more detail in the manuscript **V**. Briefly, the cells were seeded on 6-well plates and harvested after 24 hours into cold Radioimmune Precipitation Assay (RIPA) buffer. Lysates were incubated at 4°C with rotation for an hour, with subsequent clearance of insoluble material by centrifugation. Protein samples (20 μg) were electrophorezed through 7% polyacrylamide gels and transferred to a nitrocellulose membrane, which was blocked with 5% Bovine Serum Albumin (BSA). After washing, membranes were incubated for one hour with horseradish peroxidase (HRP)-conjugated secondary antibody (Dako, Glostrup, Denmark), and the signals were detected with SuperSignal West Pico chemiluminescent substrate. The primary antibodies were anti-EGFR monoclonal antibody (D38B1, Cell Signaling Technology, Danvers, MA, USA) and anti-α-tubulin monoclonal antibody (B-1-5-1-2, Sigma).

To study the cytotoxic effects of EGFR-targeted antibodies on CRC cells, cell viability assays were performed. The cells were exposed to 0-200 μg/ml cetuximab or panitumumab for 72 hours, each treatment being performed in triplicate and the experiment being repeated four times. CellTiter 96® AQueous One Solution Cell Proliferation assay was used for cell viability assessment, and the colorimetric reactions were read using a Victor2 1420 multilabel counter.

4.8. Statistical analyses

Statistical analyses were run using PASW Statistics® 18.0.1 (SPSS Inc., Chicago, IL) and IBM® SPSS® 19.0.1 (IBM Corporation, Somers, NY) software packages for Windows. For study V, SAS 9.2 and Enterprise Guide 4.2 programs (SAS Institute Inc, Cary, NC) were used for clinical data, and Microsoft Excel 2011 and StatPlus:mac LE (version 2009, AnalystSoft Inc.) for cell viability assays.

Frequency tables were analyzed with the χ^2 test, with the likelihood ratio (LR) or Fisher's exact test for categorical variables; 2x2 tables were used to calculate odds ratio (OR), and 95% confidence interval (95% CI) was defined using the exact method. Fisher's exact test, Spearman's correlation, and LR were used to assess correlation in univariate analysis. In a comparison of mean values of normally distributed variables between the treatment groups, analysis of variance (ANOVA) was utilized, whereas non-parametric tests (Mann-Whitney, Kruskal-Wallis) were used for other variables. The difference in *EGFR* GCN values obtained by different evaluation methods (normally distributed variables) were performed with the Student's t-test. The significance between the differences in the responses of the cell lines was analyzed using two-way ANOVA followed by multiple t-tests. Wilcoxon signed-ranks test was used for pairwise comparison of biomarker expression within a sample (study I) or between biopsy and operative sample (studies II, IV). Inter-observer reproducibility of the assessments was evaluated with weighted kappa, calculated with the intra-class correlation coefficient (ICC) test, in parallel mode with a two-way random model, using consistency assumption and the average-measures option to interpret the ICC (95% CI).

Univariate survival analyses for disease-free survival time (DFS; the time from diagnosis until disease recurrence or latest follow-up) and disease-specific survival time (DSS; the time from diagnosis until death from CRC or latest follow-up) based on Kaplan-Meier method, where stratum-specific outcomes were compared using log-rank statistics. In the study V, progression-free survival (PFS) was calculated from the onset of EGFR-targeted treatment until disease progression, and overall survival (OS) from the onset of EGFR-targeted therapy until death. To adjust for the covariates, a Cox proportional hazards regression model was used. Covariates that were significant in the univariate analyses were entered in a stepwise backwards manner, and are listed in the publications. CRM and vascular invasion were not included in the model due to incomplete data. All statistical tests were two-sided and declared significant at a *P*-value of <0.05.

5. RESULTS

Disease-free survival and disease-specific survival of the rectal cancer cohort (II-IV) according to disease stage at the time of operation are presented in **Figure 9.** Disease recurrence was seen in 70 patients (31%). In 18 (26%) of these cases, only local recurrence was seen, while in 52 (74%) cases, metastases to other sites or organs were found with or without local recurrence. Median time to recurrence was 16.1 months. Median follow-up time of the patients in October 2012 was 53.1 months (1.6-143.7). At the time of latest follow-up (October 2012), disease outcome was as follows: 124 (55%) patients alive without recurrence, nine (4%) patients alive with recurrence, 60 (26%) patients died of rectal cancer, and 34 (15%) patients died of other causes.

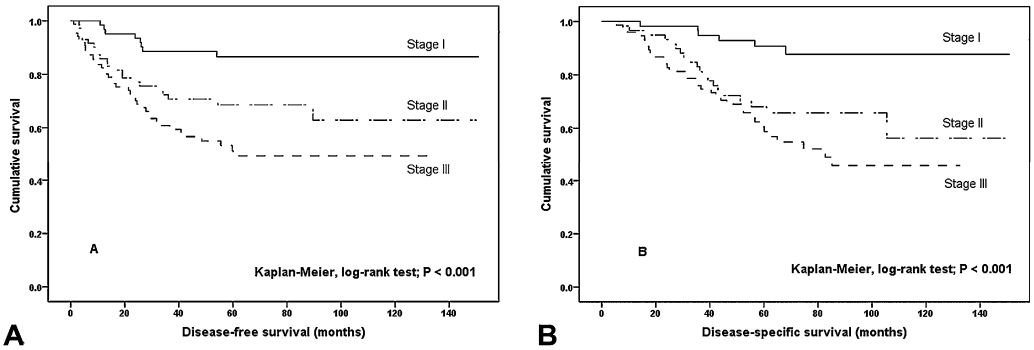

Figure 9. Disease-free survival (A) and disease-specific survival (B) according to disease stage in October, 2012.

The number of harvested lymph nodes was not dependent on the type of preoperative treatment (*P*=0.1), whereas fewer nodes were examined after APR compared to AR (*P*=0.007). Among the long-course (C)RT group, TRG after RT was poor in 27 cases (49%), moderate in 15 cases (27%) and excellent in 13 (24%) tumors. These three groups presented with divergent disease outcome, as presented in the publication **II.** Among the 13 cases with excellent response, four were T0 in pathologic examination, but in one of these cases metastasizing to regional lymph nodes was detected. Thus, the rate of pCR in our material was 5%. No one of the three patients with pCR had died of rectal cancer until October, 2012. Histological examples of TRG are presented in **Figure 10.**

5.1. IIA PLA2 expression alters during adenoma-carcinoma sequence (I)

As presented in **Figure 11**, significant differences were seen between hyperplastic polyps, adenomas, and carcinomas in terms of the number of IIA PLA2-positive cases, as well as

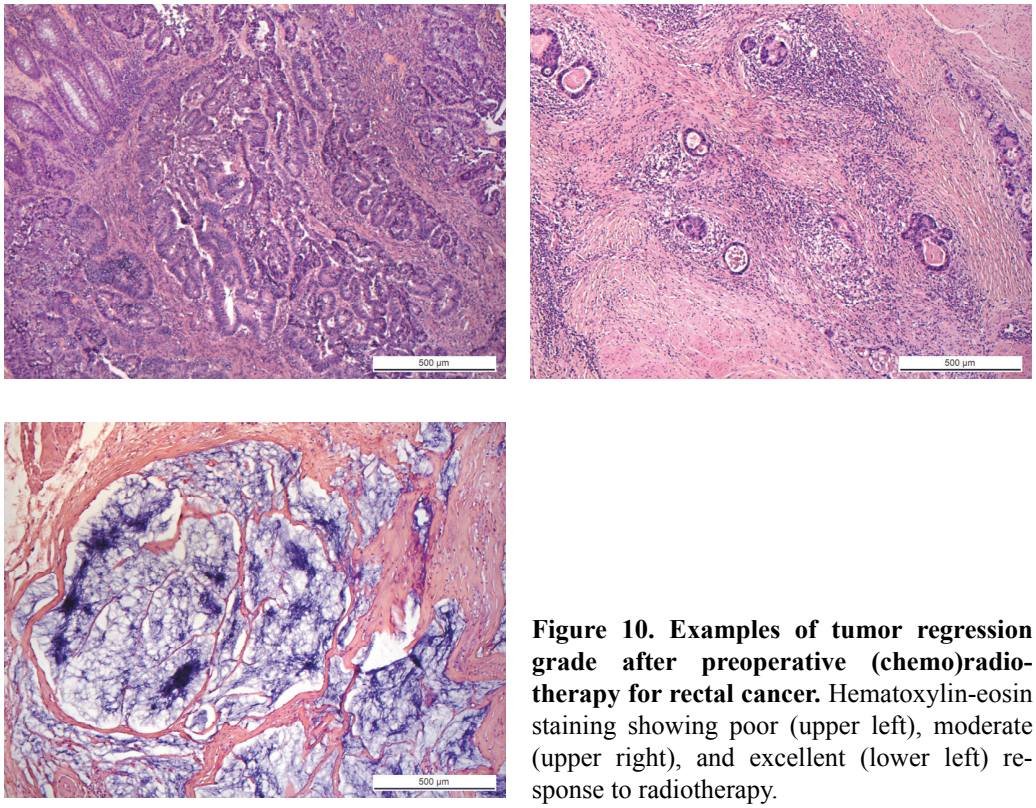

Figure 10. Examples of tumor regression grade after preoperative (chemo)radiotherapy for rectal cancer. Hematoxylin-eosin staining showing poor (upper left), moderate (upper right), and excellent (lower left) response to radiotherapy.

the mean percentage of positive immunostaining and mRNA expression**.** The results were similar in the cases where adenoma and carcinoma were present within the same sample. Among these ten cases, adenomas showed IIA PLA2-immunopositive cells in each sample as compared to carcinomas that showed positivity in only one third of the cases, and with significantly (P<0.001) less percentage of positive cells compared to adenomas. Colonic and rectal tumors showed no differences in their IIA PLA2 expression patterns.

ISH affirmed that IIA PLA2 mRNA was synthesized in the same epithelial cells showing IIA PLA2 protein in IHC. Protein and mRNA expression of IIA PLA2 correlated closely (R=0.93, P<0.001) with each other. When present, IIA PLA2 protein (IHC) was usually localized in the apical cytoplasm of the tumor epithelial cells. In 44% of carcinomas, IIA PLA2-immunopositive cells were seen in the invasive tumor front. In addition, IIA PLA2 protein was found in apoptotic and necrotic cells, and in normal colorectal epithelium adjacent to carcinoma. Normal colorectal mucosa in immediate proximity to carcinoma was IIA PLA2-immunopositive in most of the cases (96%), and compared to normal mucosa situated further from carcinoma, it was presented with stronger staining intensity (P=0.012).

Among the 72 rectal carcinomas of the second set, the amount of IIA PLA2 expression tended to increase along with tumor differentiation grade, but the difference was not statistically significant (P=0.09). A higher number of immunopositive cells was

seen in tumors with an excessive mucinous component as compared to conventional adenocarcinomas (*P*=0.025). IIA PLA2 immunopositivity was inversely correlated with the percentage of Ki-67 expression that was analyzed in the study II (R= -0.25; *P*=0.03). No other correlations were seen between IIA PLA2 protein and clinicopathologic features. DFS and DSS of the patients did not differ according to IIA PLA2 expression (positive *vs* negative; invasive front positive *vs* negative; *unpublished data*).

	Hyperplastic	Adenomas	Carcinomas
[a] **IHC+**	0/7	19/24 (79%)	26/83 (31%)
[a] **ISH+**	0/4	8/12 (67%)	3/9 (33%)
[b] **Mean IHC%**	0	14.8	0.8
[b] **Mean ISH%**	0	5.0	1.3
[c] ***P*-value**	0.001 (*vs* adenoma)	<0.001 (*vs* carcinoma)	

[a] IHC+ and ISH+ indicate the number of cases with over 1% of positive tumor cells.
[b] Mean ICH% and ISH% indicate the mean percentage of IIA PLA2-positive epithelial cells in IHC and ISH. ISH was performed in four hyperplastic polyps, 12 adenomas, and nine carcinomas.
[c] Mann-Whitney *U* test for differences between the mean percentage of IIA PLA2-positive cells in IHC.

Figure 11. IIA PLA2-positive hyperplastic polyps, adenomas, and carcinomas.

5.2. Securin protein expression is decreased after radiotherapy and may indicate poor outcome in a subset of patients (II)

Almost every biopsy (98% for securin; 100% for Ki-67) and operative (98% for securin; 99% for Ki-67) sample showed positive immunostaining for securin and Ki-67. The protein expression of securin and Ki-67 correlated closely (R=0.38, *P*=0.004 for biopsies; R=0.47, *P*<0.001 for operative samples). Compared to securin, the mean percentage of Ki-67 expression was higher both in biopsy (47% *vs* 83%) and operative (31% *vs* 56%) samples (*P*<0.001 for both comparisons).

A detailed description of securin and Ki-67 protein expression as related to clinicopathologic variables is presented in the respective publication. When analyzing the whole study population, patients over 70 years of age had higher expression of both markers than patients under 70 years of age (*P*=0.004 for securin; *P*=0.02 for Ki-67). Regarding securin, protein expression was higher in superficial (T1-2) tumors and decreased along with depth of invasion (*P*=0.01).

Securin and Ki-67 protein expression in relation to preoperative treatment are depicted in **Figure 12.** Neither biomarker, as analyzed from pre-irradiation biopsy

samples, was predictive for TRG. The percentage of Ki-67 expression in post-irradiation operative samples inversely correlated with TRG (*P*=0.02). The same tendency of securin expression did not reach statistical significance in three-point analysis of TRG (*P*=0.21) but approximated significance when moderate and excellent response were studied together against poor response (*P*=0.05).

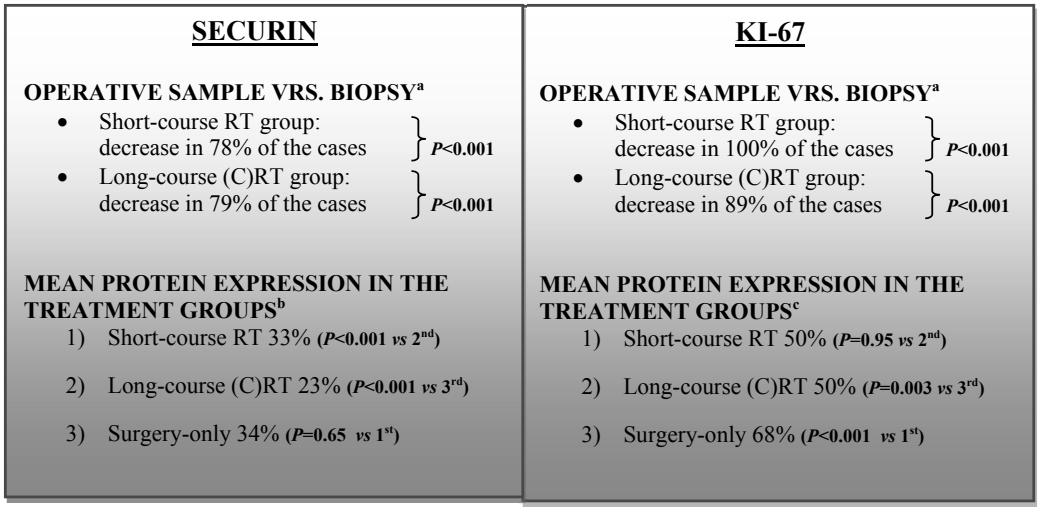

Figure 12. Securin and Ki-67 related to preoperative treatment. Statistical analyses performed using [a] Wilcoxon signed-ranks test; [b]ANOVA; [c] Kruskal-Wallis test.

Securin and Ki-67 were not related to disease outcome when analyzing the whole study population, or when examining patients in the short-course RT and surgery-only groups. Instead, high securin protein expression (over median value defined for each treatment group) in operative sample was a prognosticator for adverse DSS in the long-course (C)RT group (*P*=0.019). After adjustment to sex, age, postoperative T and N, disease recurrence and TRG, high securin expression remained as an independent adverse prognostic factor (HR=5.3; 95%CI 1.1-25.0; *P*=0.036) for DSS together with disease recurrence (HR=24.3; 95%CI 2.2-269.8; *P*=0.009) and patient age (HR=1.1; 95%CI 1.0-1.2; *P*=0.043). When disease recurrence was withdrawn from the model, the independent adverse prognostic factors for DSS were lymph-node positive disease (for N2 *vs* N0, HR=7.27; 95%CI 1.08-48.96; *P*=0.041) and high securin expression (HR=9.96; 95%CI 1.73-57.17; *P*=0.010).

5.3. Intratumoral location of CD44v6 protein contributes to invasive potential (III)

The results of membranous and cytoplasmic CD44v6 stainings are shown in **Table 14.** Of the 210 samples, 84% were positive for membranous and 81% were positive for cytoplasmic

immunostaining. Membranous and cytoplasmic CD44v6 staining correlated both in terms of percentage (R=0.4; $P<0.001$) and intensity (R=0.31; $P<0.001$) of expression. Patients in the long-course (C)RT group showed less cytoplasmic CD44v6 protein expression in their tumors as compared to patients in the surgery-only group ($P=0.002$). Generally, the percentage or intensity of CD44v6 expression did not correlate with clinicopathologic variables or disease outcome. Using pan-cytokeratin staining and Jass' classification (Jass *et al*. 1986), 48% of tumors showed expanding and 52% infiltrating growth pattern.

The location of CD44v6 protein expression in the 177 tumors with positive membranous immunostaining was as follows: mainly in the central tumor parts in 72 cases; mainly in the invasive tumor front in ten cases; and equally in central parts and invasive front in 95 cases. Accordingly, 72 cases were appointed as having "front-negative" and 105 cases as having "front-positive" staining pattern of membranous CD44v6. Compared to front-positive tumors, front-negative pattern associated with smaller CRM ($P=0.01$), infiltrating tumor growth pattern ($P<0.001$) and increased risk of recurrence ($P=0.01$).

In univariate survival analysis, patients with front-negative tumors presented with significantly shorter DFS ($P=0.022$), and the same tendency was preserved in subgroup analysis of the short-course RT ($P=0.058$) and surgery-only ($P=0.024$) treatment groups, but not in the long-course (C)RT group. DSS was not significantly different between patients with front-positive and -negative tumors, but the statistical association was nevertheless stronger with the updated follow-up data (October, 2012; $P=0.15$) than with data in the original publication ($P=0.68$). In univariate analysis, infiltrating growth pattern tended to relate to shorter DFS ($P=0.015$) and DSS ($P=0.14$) as compared to expanding type. As presented in the publication**,** none of the variables (location of membranous CD44v6 staining, tumor growth pattern) was found to be an independent prognostic factor for rectal cancer outcome when adjusted to other clinicopathologic characteristics.

Table 14. CD44v6 expression in rectal cancer.

Percentage of membranous immunostaining [a]	***n*=210**	**Percentage of cytoplasmic immunostaining** [a]	***n*=210**
<5%	79	<5%	67
5-20%	72	5-20%	63
21-50%	34	21-50%	59
>50%	25	>50%	21
Intensity of membranous immunostaining [b]	***n*=210**	**Intensity of cytoplasmic immunostaining** [b]	***n*=210**
Negative	33	Negative	40
Weak	85	Weak	114
Moderate	71	Moderate	50
Strong	21	Strong	6

For statistical purpose, [a] Tumors with immunopositivity ≤20% were studied as one group and those with immunopositivity >20% as another group; [b] negative and weak staining intensities were studied as one group and moderate and strong as another group.

5.4. ALDH1 is an adverse prognosticator in node-negative rectal cancer (IV)

ALDH1 immunopositivity above cut-off value (3%) was seen in 55% of the biopsy samples and 71% of the operative samples. Among immunopositive biopsies, staining intensity was weak in 69% of the cases, and moderate or strong in 31% of the cases. In operative samples, the respective percentages were 42% and 58%. ALDH1 expression did not relate to the common clinicopathologic variables, including TRG. The association of ALDH1 protein expression with nuclear β-catenin expression pattern is presented in **Table 15.** In addition, ALDH1 expression in biopsy specimens inversely correlated with Ki-67 expression in biopsies (R= -0.3; *P*=0.03).

The pairwise comparisons of ALDH1 immunostaining between pre-irradiation biopsy samples and post-irradiation operative samples are presented in the respective publication**.** Briefly, the comparison was performed using two methods: 1) positive/ negative ALDH1 immunostaining and 2) staining intensity of ALDH1. With both approaches, protein expression was remained stable or upregulated in most of the cases in response to RT, while downregulated in few cases only (*P*=0.02 for positive/negative category; *P*<0.001 for staining intensity category). Similarly, in a comparison of the three treatment groups, ALDH1 expression was more often positive in the two RT-groups as compared to the surgery-only group (*P*=0.04).

Table 15. The relation of ALDH1 immunostaining with nuclear β-catenin expression pattern in 197 cases.

	Conserved regulation of nuclear β-catenin (*n*=116)	**Deficient regulation of nuclear β-catenin (*n*=81)**	*P* [a]
ALDH1			
Negative (*n*=54)	39 (34%)	15 (19%)	0.018
Positive (*n*=143)	77 (66%)	66 (81%)	
ALDH1 intensity			
Negative/weak (*n*=115)	77 (66%)	38 (47%)	0.006
Moderate/strong (*n*=82)	39 (34%)	43 (53%)	

[a] Statistical significance with likelihood ratio.

As presented in **Figure 13**, high ALDH1 expression in biopsy samples tended to predict shorter DFS (*P*=0.09) and DSS (*P*=0.08) as compared to ALDH1-negative biopsies (univariate analysis). A similar tendency for DSS was seen concerning ALDH1-positivity in operative samples (*P*=0.17). Among node-negative patients, positive ALDH1 expression in operative samples indicated shorter DFS (*P*=0.038) and DSS (*P*=0.049). Node-negative patients treated with postoperative chemotherapy treatment were further examined as an entity. As presented in **Figure 14**, ALDH1-positivity in

operative samples associated with shorter DFS (*P*=0.078) and DSS (*P*=0.031) in this group of patients. After adjustment to patient age (continuous variable), sex, preoperative treatment (with or without RT) and postoperative stage (stage I or II), positive ALDH1 expression remained as an independent adverse prognosticator for DFS (*P*=0.044) and DSS (*P*=0.049). Other independent prognostic factors are enumerated in the respective publication**.**

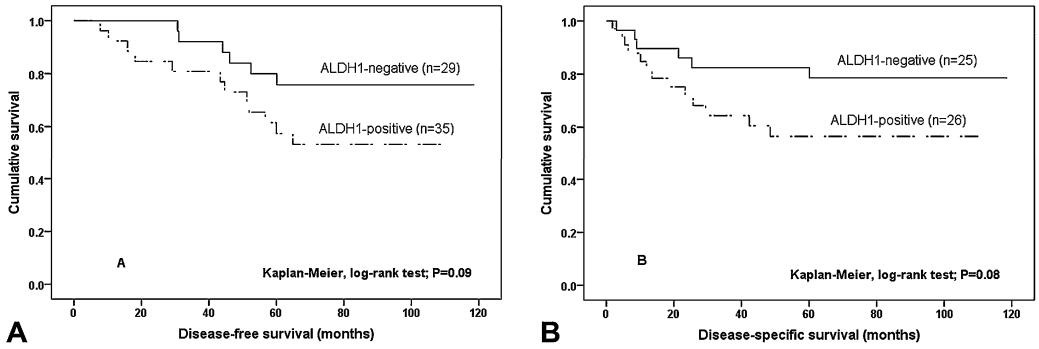

Figure 13. Disease-free (A) and disease-specific (B) survival time according to ALDH1 expression in pre-irradiation biopsy samples.

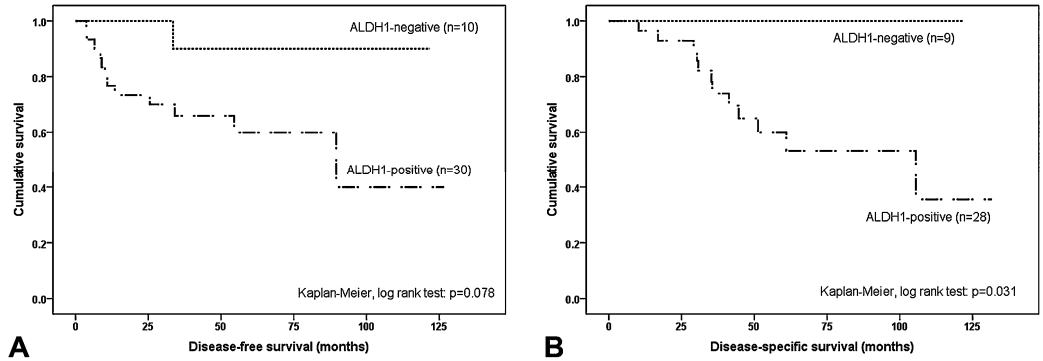

Figure 14. Disease-free (A) and disease-specific (B) survival time according to ALDH1 expression in node-negative rectal cancer patients treated with postoperative chemotherapy.

5.5. Heterogeneous *EGFR* gene copy number increase predicts response to anti-EGFR treatment in metastatic colorectal cancer (V)

Altogether 29 tumors in the original cohort (64%) and 18 tumors in the validation cohort (58%) showed increased *EGFR* GCN (GCN≥4). No statistically significant difference was seen in *EGFR* GCN between colonic and rectal tumors (*P*=0.14). As presented in

Table 16, increased GCN related to improved rate of clinical benefit from cetuximab and panitumumab, and superior PFS and OS both in the validation cohort and in the combined analysis of the original and validation cohorts. Similar results were achieved in the analysis of the chemorefractory group. The survival curves are presented in the respective manuscript**.**

The benefit from EGFR-targeted treatment according to the *EGFR* GCN in the chemorefractory group *(n*=54) was assessed separately for cetuximab and panitumumab. Two patients were treated with both antibodies, and were excluded from the analysis. Increased *EGFR* GCN predicted improved clinical benefit rate in the group treated with cetuximab ± chemotherapy (*n*=31; *P*=0.0007) but failed to reach statistical significance in the group treated with panitumumab ± chemotherapy (*n*=21; *P*=0.4). PFS was statistically significantly longer in both treatment groups if the *EGFR* GCN was ≥ 4 (median PFS 30 *vs* 10 weeks, *P*<0.0001 for cetuximab; median PFS 22 *vs* 14 weeks, *P*=0.03 for panitumumab), whereas OS was significantly longer only in the patients treated with cetuximab ± chemotherapy (median OS 12.5 *vs* 4.6 months, *P*=0.0006).

Table 16. Clinical benefit rate, median progression-free survival (PFS) and median overall survival (OS) according to *EGFR* gene copy number (GCN).

Cohort	Clinical benefit	*P* [a]	PFS	*P* [b]	OS	*P* [b]
Validation (*n*=31)						
· GCN<4 (*n*=13)	31%	0.009	11 wk	0.002	8.2 mo	0.004
· GCN≥4 (*n*=18)	78%		25 wk		12.1 mo	
Combined (*n*=76)						
· GCN<4 (*n*=29)	27%	<0.0001	11 wk	<0.0001	7.8 mo	0.0005
· GCN≥4 (*n*=47)	80%		30 wk		16.4 mo	
Combined chemo-refractory (*n*=54)						
· GCN<4 (*n*=21)	32%	0.0004	11 wk	<0.0001	7.2 mo	0.0002
· GCN≥4 *(n*=35)	80%		30 wk		12.5 mo	

Statistical significance with [a] chi-square and [b] log-rank tests.

The mean *EGFR* GCN was significantly lower when the *EGFR* GCN was analyzed in a random way without IHC guidance, as compared to the method where the cells with the highest *EGFR* GCN were chosen for analysis (*P*<0.0001). Median GCN using the former method was 3.3 while it was 4.3 using the latter method, respectively. When the values obtained from random analysis were used to predict benefit from EGFR-targeted treatment, no statistically significant differences were seen in clinical benefit rate, PFS, and OS according to *EGFR* GCN (<4 *vs* ≥4).

In the cell lines, *EGFR* GCN correlated positively with the amount of EGFR protein, as shown with Western blotting. The responses to EGFR-targeted therapy differed significantly between the four cell lines (P=0.00002 for cetuximab; P= 0.00034 for panitumumab), the C2BBe1 cell line with *EGFR* GCN 4 and WT *KRAS* being the most responsive to both drugs ($P<0.001$ compared to other cell lines). With a 200 μg/ml treatment with either of the antibodies, the viability of the cells was about 60% compared to non-treated control cells (63% for cetuximab, $P<0.001$; 64% for panitumumab, $P<0.001$). The cell line with *EGFR* GCN 2 and WT *KRAS* (CW-2) appeared to be most resistant to cetuximab and panitumumab, while the cell lines with mutant *KRAS* and *EGFR* GCN >4 (NCI-H747, SK-CO1) showed intermediate sensitivity.

6. DISCUSSION

CRC is one of the leading causes of cancer deaths worldwide. It is an optimal target of studying the progression of premalignant lesions to malignant tumors as the preceding lesion in most of the cases is known to be an adenoma. The treatment decisions of CRC are mostly based on the disease stage at the time of diagnosis and operation. However, a significant variation in outcome is seen even within the stages. Consequently, tissue-based prognostic factors are required to guide more individualized treatment decisions and to optimize disease outcome. Similarly, predictive factors would be of indispensable value in determining whether a patient benefits from radiotherapy, chemotherapy, and biologic treatments.

In the present thesis, the expression of selected biomarkers was studied in different phases of colorectal carcinogenesis. First, IIA PLA2 expression was examined to detect possible changes in expression during the progression of premalignant lesions to malignant tumors. In a material consisting of primarily non-metastasized rectal tumors, the protein expression of selected biomarkers was analyzed in relation to preoperative treatment and disease prognosis. Finally, a set of metastasized colorectal tumors was studied in order to validate the predictive value of *EGFR* GCN on clinical benefit from EGFR-targeted antibodies. The biomarkers in question were selected because interesting, but conflicting, findings have been reported about their contribution to colorectal carcinogenesis, progression, and treatment response. In addition, the examined biomarkers are often involved in the same hallmarks of cancer as shown in **Figure 4,** and several interrelations exist between their signaling pathways. For instance, IIA PLA2 is related to EGFR- (Hernández *et al.* 2010) and Wnt/β-catenin signaling (Ganesan *et al.* 2008), which in turn contribute to cellular proliferation (Spano and Vignot, 2007; Yao *et al.* 2011) together with Ki-67 (Gerdes *et al.* 1984) and securin (Jallepalli *et al*. 2001) activity.

6.1. IIA PLA2 (I)

IIA PLA2 is an inflammatory mediator with both pro- and anti-inflammatory functions (Fijneman and Cormier, 2008). It functions in several pathways implicated in intestinal tumor development (Fijneman and Cormier, 2008), but the data concerning its protein expression and site of synthesis in CRC are conflicting (Edhemovic *et al*. 2001; Buhmeida *et al*. 2009) or incomplete. We hypothesized that IIA PLA2 expression might alter during adenoma-carcinoma sequence of CRC, and that the amount of expression might distinguish prognostically different groups of patients. In the study I, we showed that IIA PLA2 mRNA and protein are present in the same epithelial cells and that the expression is significantly different between benign, premalignant and malignant colorectal lesions. However, IIA PLA2 expression did not relate to disease outcome.

Hyperplastic polyps have traditionally been considered as benign lesions with no malignant potential, although they have also been suggested to have some premalignant potential in the case of sporadic MSI-H CRC (Hawkins and Ward, 2001). Adenomas, in turn, are explicitly contributed to malignant potential. We found each of the hyperplastic polyps to be devoid of IIA PLA mRNA and protein, whereas most of the adenomas showed immunopositive cells in IHC and ISH. This is supported by earlier findings of Kennedy *et al*. (1998). Conversely, the amount of immunopositive malignant tumors was smaller than that of premalignant adenomas, and when present, the number of IIA PLA2 positive cells was rather small. Similar results were seen in the ten samples with adenoma and carcinoma in the same sample. Our results suggest that malignant CRC cells may lose their ability to express IIA PLA2, and that IIA PLA2 may participate remarkably in colonic carcinogenesis. Although *IIA PLA2* gene has not been demonstrated to be a major tumor-suppressor gene in human in contrast to situation with mice (MacPhee *et al.* 1995; Cormier *et al.* 1997), IIA PLA2 expression has been associated with favorable outcome among others in gastric cancer (Xing *et al.* 2011). The protective role of IIA PLA2 expression might relate to its ability to degrade intestinal microbes and to regulate normal intestinal flora (MacPhee *et al.* 1995). Moreover, IIA PLA2 could have growth-suppressing actions as its expression was inversely correlated with proliferative activity of rectal carcinomas in our study. At the same time, a pro-proliferative function could be expected because IIA PLA2 has been demonstrated to induce EGFR-signaling in brain tumors (Hernández *et al*. 2010). Thus, the actual implication of IIA PLA2 in cellular proliferation needs further studies. Finally, IIA PLA2 could protect against cancer via its metabolite arachidonic acid that is known to induce apoptosis (Cao *et al.* 2000). Agreeing with this hypothesis, we found apoptotic and necrotic cells rather often to express IIA PLA2 which could, however, also reflect the preference of IIA PLA2 to degrade perturbed cell membranes (Leidy *et al*. 2006).

Our findings of minimal or absent presence of IIA PLA2 protein in malignant colorectal tumors are in accordance with Edhemovic *et al.* (2001) but contradict those of some others (Buhmeida *et al.* 2009) reporting more pronounced immunopositivity in CRC. As for clinical outcome and IIA PLA2, we did not find any differences in disease outcome according to IIA PLA2 immunopositivity, although it has previously been related to adverse survival in CRC (Buhmeida *et al.* 2009) and beneficial survival in prostatic (Mirtti *et al.* 2009) and gastric (Xing *et al.* 2011) cancer. The controversy between our results and those of Buhmeida *et al.* could relate to differences in disease stages, antibodies, scoring systems, and tumor sites, albeit we did not detect differences in IIA PLA2 immunopositivity between colon and rectal tumors. The tissue samples in our series were stained with an inhouse antibody with no significant cross-reactivity with other secretory PLA2s (Grönroos *et al.* 2002; Nevalainen *et al.* 2005), and a commercial antibody was used only for a validation intent. Furthermore, the sample size for survival analyses was relatively small, as only the rectal carcinomas without any preoperative treatments were included.

Peritumoral healthy mucosa showed increased immunopositivity for IIA PLA2 protein in virtually all of the cases, in accordance with earlier reports (Edhemovic *et al*. 2001; Buhmeida *et al.* 2009). This phenomenon most probably results from the secretion of pro-inflammatory cytokines from the carcinoma tissue. The same mechanism might contribute to the frequent presence of IIA PLA2 immunopositive cells in the tumor invasive front. In addition to cancer cells, pro-inflammatory cytokines are secreted by macrophages of the surrounding stroma, potentially explaining why some cases with no evident expression in the major tumor bulk still presented with IIA PLA2 protein in the invasive tumor front. Moreover, cells in the invasive front often presented with a disintegrated appearance, which could attract the hydrolyzing function of IIA PLA2. Our findings are in accordance with Tribler *et al.* (2007) reporting increased expression of IIA PLA2 in peripheral parts of the carcinoma as compared to more central location.

One of the limitations of our study was the rather small number of malignant tumors enrolled. This may have contributed to the lack of differences in clinical outcome according to tumor IIA PLA2 expression status, as well as to lack of statistically significant correlations between common clinicopathologic variables and IIA PLA2 expression. Nevertheless, the main aim of this study was to compare the presence and expression of IIA PLA2 between benign, premalignant and malignant lesions, and for this reason we preferred not to include preoperatively irradiated tumors into this study. We also acknowledge that IIA PLA2 mRNA might be less stable molecule compared to IIA PLA2 protein, and that chemical treatment with formalin may affect the ISH data generated from paraffin-embedded tissue material. Our method, however, was based on RNAase-free conditions, and protein and mRNA most often were shown to be expressed in the same cells (epithelial cells). Considering these issues together with the previous experience with the same antibody (Haapamäki *et al.* 1997), we believe that the ISH results of our study were reliable.

In conclusion, IIA PLA2 expression is decreased in malignant CRC compared to premalignant adenomas. Accordingly, downregulation of IIA PLA2 may have an important biological role during colorectal carcinogenesis. In the future, it would be interesting to study the expression of this molecule in irradiated tumors as well, because IIA PLA2 has preference to degrade perturbed cellular membranes.

6.2. Biomarkers in rectal cancer material (II, III, IV)

Ki-67, securin, CD44v6, β-catenin and ALDH1 protein expression were analyzed in rectal carcinomas. All of these biomarkers have been associated with cellular proliferation (Gerdes *et al.* 1984; Jallepalli *et al*. 2001; Yao *et al.* 2011; Yoshida *et al.* 1992), and securin gene (*PTTG1*), β-catenin, ALDH1, and EGFR expression also with cancer stem cell function (Yoon *et al*. 2012; Yao *et al.* 2011; Ginestier *et al.* 2007; Feng *et al.* 2012). We hypothesized that the expression status of these markers is altered in

response to RT, and that they might be of value in predicting the disease outcome and benefit from RT in rectal cancer. The strengths of the studies were the inclusion of only stage I-III carcinomas of either low or middle rectum to achieve a fairly homogenous study material, and the use of whole-tissue sections instead of tissue microarray (TMA) methods, thus enabling us to scrutinize protein expression in a larger tumor area including both invasive front and central parts of a tumor. Furthermore, securin and ALDH1 were studied together with more conventional proteins (Ki-67, β-catenin) that are at least partially contributed to same hallmarks of cancer with them. All the immunostainings were analyzed individually by two observers with good inter-observer reproducibility values.

Some weaknesses of the material and studies need closer examination. First, the three treatment groups were divergent in terms of some basic clinico-pathologic features (patient age, tumor stage and CRM). This is inevitable considering the clinical guidelines of rectal cancer treatment (Schmoll *et al*. 2012) and the retrospective study design. There also were missing data concerning lymphovascular invasion and CRM. We did not include these prognostic factors into multivariate analysis because this would have markedly abridged the number of cases in the model. The effect of (C)RT on protein expression was analyzed using two approaches. First, pre-irradiation biopsy sample was compared to operative sample, and second, tumors in surgery-only group were compared to those in the short- and long-course (C)RT groups. The biopsies may sometimes be small-sized with rather scanty presentation of malignant cells. Although we excluded biopsies with very small number of tumor glands, over- and underestimation of protein expression cannot be completely excluded.

6.2.1. Securin and Ki-67 (II)

PTTG1 and its transcriptional product, securin, participate in several cellular functions including proliferation (Zou *et al.* 1999; Jallepalli *et al.* 2001), apoptosis (Yu *et al.* 2000; Bernal *et al*. 2002), malignant transformation (Pei and Melmed, 1997), tumor invasiveness (Heaney *et al.* 2000; Yoon *et al*. 2012), and the fate of cells in response to DNA-damaging agents (Chen *et al.* 2010). A single mutation in *PTTG1* has been shown to be sufficient to induce oncogenic properties of securin (Mora-Santos *et al.* 2012). Increased expression of securin is already seen in precancerous adenomas implicating that it may have a role in relatively early phases of carcinogenesis (Heaney *et al.* 2000). This view is further supported by the correlation of securin expression with that of aberrant nuclear β-catenin, which is considered as one of the earliest tumorigenic events in colorectal carcinogenesis (Hlubek *et al.* 2006). In the study II, we analyzed securin protein expression in rectal cancer together with that of Ki-67, and demonstrated that the expression of both markers was decreased after preoperative (C)RT, and that high securin expression (above median) after long-course (C)RT independently predicted adverse disease-specific outcome.

Both depletion and overexpression of securin may result in dysregulated cellular proliferation (Vlotides *et al.* 2007) which has given rise to questions on its actual complicity in cellular proliferation. On one hand, securin is required to inhibit premature sister chromatid separation during mitosis, and on the other hand its degradation is necessary to allow mitosis to proceed (Jallepalli *et al.* 2001). In accordance with previous studies by Filippella *et al.* (2006) and Hlubek *et al.* (2006), we found securin expression to correlate with Ki-67 expression, strengthening the view of securin as a proliferation inducing protein. Despite correlation, the amount of securin protein was lower than that of Ki-67, which most probably reflects diverse and somewhat complex implications of securin in other cellular processes, such as apoptosis. Ki-67, in turn, has mostly been related to proliferative function only.

In a comparison of securin and Ki-67 expression between pre-irradiation biopsy sample and post-irradiation operative sample, both markers showed decreased expression in response to (C)RT. This is well reasonable, because proliferating cells are most sensitive to RT (Pawlik *et al*. 2004). Concerning securin, our study is the first one to report such a finding in human tissues, whereas similar results have been shown with regard to Ki-67 (Debucquoy *et al*. 2009). However, when expression of securin and Ki-67 between the three treatment groups was compared, the findings were less explicit. Securin expression in the short-course RT and surgery-only groups was surprisingly similar, as was Ki-67 expression in the two RT-groups. This might relate to cellular kinetics in response to irradiation. The interval between the end of long-course (C)RT and surgery is several weeks, which may lead to compensatory cellular repopulation (Denekamp, 1986), thus explaining the lack of further decrease in Ki-67 expression after this treatment modality. As to securin, repopulation effect might be less relevant because securin is involved in apoptosis (Yu *et al.* 2000; Bernal *et al.* 2002) which, in turn, is one of the main mechanisms how RT executes its effects (Pawlik *et al*. 2004). Short-course RT might have less effect on the level of securin expression because the time interval between the RT and operation is only one week.

Ki-67 expression in pre-irradiation biopsy samples was not predictive for tumor regression grade after RT. In the previous studies, results have been highly controversial (Willett *et al*. 1995; Jakob *et al*. 2008; Terzi *et al.* 2008), possibly relating to the relatively small size of biopsy samples. This may lead to over- or underestimation of the tumor proliferative activity, especially considering the reported heterogeneity in expression of proliferation markers in CRC (Kressner *et al.* 1995). Accordingly, analysis of the average level of proliferation activity may be insufficient to mirror CRC heterogeneity. In the case of securin, our study was the first one to evaluate the predictive value of securin on rectal cancer RT response as the other studies have been conducted with cell lines (Chiu *et al.* 2007; Chen *et al*. 2010). In these studies, securin expression has been related to maintenance of genomic stability (Bernal *et al*. 2008), radiosensitivity, and fate of CRC cells (Chen *et al*. 2010) after DNA-damage. Our results do not support this view, as pre-irradiation securin expression did not relate to TRG after RT. The diverse, and in some

cases controversial, complicity of securin in cellular proliferation, apoptosis, and DNA damage response might complicate its interpretation as a predictive biomarker for rectal cancer preoperative RT, although the lack of correlation can also reflect the rather small number of biopsies in our study.

We did not find high securin expression to correlate with lymph node metastases in contrast to earlier reports in CRC (Heaney *et al.* 2000; Wu *et al.* 2008). In turn, differences in postoperative tumor depth of invasion and patient age were seen according to securin and Ki-67 expression. These differences, however, most probably reflect the effect of RT on the studied markers as the two parameters diverged between the treatment groups in the first place. Importantly, high securin expression after long-course (C)RT was an independent prognosticator of adverse DSS. No such association was seen in other treatment groups for securin, or any treatment groups for Ki-67. The importance of securin in this given treatment group might relate to the finding that depletion of securin impairs DNA repair, increasing DNA damage and senescence (Chen *et al.* 2010). Most of the patients in the long-course RT group received 5-FU based chemotherapy as a radiosensitizer, and often were treated with postoperative adjuvant chemotherapy. Thus, high securin expression after long-course (C)RT might enable DNA repair and continuation of cell cycle instead of cellular death. The number of long-course (C)RT patients in our study, however, was rather small to come to any further conclusion. Moreover, securin has paradoxically demonstrated to be capable of interacting with and inhibit DNA repair proteins such as p53 and Ku70 (Bernal *et al*. 2002; Kim *et al.* 2007).

Taken together, securin and Ki-67 expression are decreased after RT for rectal cancer. The diverse cellular functions of securin likely explain the somewhat different expression profile of these markers despite their mutual correlation. High securin expression after long-course (C)RT indicates shorter DSS, potentially reflecting the functions of securin in DNA repair and cellular senescence. Patients with high securin expression might need more aggressive treatment approaches after operation for rectal cancer.

6.2.2. CD44v6 (III)

CD44v6 is a variant transcript of CD44-family of glycoproteins that binds and presents growth factors, and mediates cell-cell and cell-matrix interactions (Lesley *et al.* 1993; Bennett *et al.* 1995; Misra *et al*. 2011). CD44v6 is overexpressed in CRC (Gorham *et al*. 1996) but its prognostic value is widely ambiguous (Ropponen *et al*. 1998; Zlobec *et al*. 2009). This might be explained by the inclusion of both colonic and rectal tumors in most of the studies, because CD44v6 has been demonstrated to be differently expressed between these two locations (Minoo *et al.* 2010). In the studies including solely rectal tumors (Peng *et al*. 2008; Zhu *et al.* 2010), the biomarker has not been systematically analyzed related to preoperative RT. In the study III, we examined CD44v6 protein expression and its intratumoral distribution in rectal cancer, and found that lack of membranous expression in the tumor invasive front associated with shorter DFS and

infiltrating tumor growth pattern. No differences between the three treatment groups were seen according to CD44v6 expression status.

CD44v6 was detected both in the cell cytoplasm and membrane, as reported also previously (Zlobec *et al.* 2009). Although suggested to associate with cellular dedifferentiation (Faleiro-Rodrigues and Lopes, 2004), the significance of cytoplasmic protein is mostly unknown (Zlobec *et al*. 2009). We found the amount of cytoplasmic staining to be smaller after preoperative (C)RT compared to cases with no preoperative treatment, which may reflect the wide-ranging histological alterations caused by RT (Nagtegaal *et al.* 2002). Even though we did not perform a pairwise comparison of expression in biopsy and operative samples, it appears that RT does not have a major effect on membranous CD44v6 protein expression, as it showed no difference between the three treatment groups. Previously, Coppola *et al.* (1998) have mentioned parallel observation.

The amount of CD44v6 protein expression did not relate to clinicopathologic variables or disease outcome in our study, which is in contrast with some earlier rectal cancer studies (Peng *et al.* 2008; Zhu *et al.* 2010). The reported heterogeneity of CD44v6 expression within a tumor (Zlobec *et al.* 2009) and during tumor progression (Bendardaf *et al*. 2006), as well as the differences in antibodies, scoring systems and disease substages between the studies are potential reasons for the conflicting results. Furthermore, taking into consideration the involvement of CD44 molecules in complex signaling networks of membrane tyrosine kinases (Orian-Rousseau *et al.* 2002), as well as their ability to act both as invasiveness-promoting and tumor-suppressing molecules (Herrlich *et al.* 2000), it becomes comprehensible that analysis of the percentage of expression may be insufficient.

In addition to amount of expression, we assessed the intratumoral distribution of membranous CD44v6. Tumor invasive front is the interface between the invading edge of a tumor and the surrounding stroma, and altered expression of several adhesion molecules are seen in this area (Brabletz *et al.* 1998; Gosens *et al*. 2007). Interestingly, the absence of membranous CD44v6 protein expression in the invasive front correlated with infiltrating tumor growth pattern, small circumferential margin and an increased risk of disease recurrence. Previously too, weaker or absent CD44v6 expression has been reported in tumors growing in a diffuse manner (Ishida *et al.* 2000; Zlobec *et al.* 2009), although these studies did not systematically assess the prognostic value of protein location within a tumor. Our study support the view of Coppola *et al*. (1998) in that a loss of membranous CD44v6 expression in the invasive front may enable invasive and metastatic spread of tumor cells due to defective binding of cancer cells to ECM. Indeed, CD44v6 has been shown to possess higher affinity to hyaluronate than standard CD44 isoform (Sleeman *et al.* 1995). Further supporting this view, loss of E-cadherin expression has been reported to correlate with loss of CD44v6 protein expression (Zlobec *et al*. 2009), indicating that insufficient CD44v6 expression in the invasive front may result in defects also in cell-cell interaction.

Absence of CD44v6 expression in the invasive front predicted shortened DFS in univariate analysis, as did infiltrating tumor growth pattern. Instead, neither remained an independent prognosticator in multivariate model, potentially indicating that these variables lack significant power to predict survival after adjustment to conventional prognostic factors. Alternatively, the mutual correlation of tumor growth pattern and intratumoral staining pattern of membranous CD44v6 might interfere the model. DSS was not significantly different in patients with front-positive and front-negative tumors, although with longer follow-up time, tended to be more favorable in the former group.

To be concluded, the location rather than the amount of membranous CD44v6 protein expression seems to be important for rectal cancer progression and outcome. Determination of the intratumoral location might also be less analyzer-dependent compared to assessment of the amount of expression. Absence of membranous CD44v6 protein expression in tumor invasive front can allow cancer cells to detach from neighbouring cells and ECM, facilitating invasion and metastasis. Patients presenting with front-negative tumors may be in a need of close monitoring and intensified therapeutic options.

6.2.3. ALDH1 and β-catenin (IV)

Cancer stem cells are considered as pluripotent cells with the ability to initiate and sustain malignant growth (Reya *et al.* 2001). Cheung *et al.* (2007) were the first to demonstrate that increased aldehyde dehydrogenase (ALDH) activity could be used to isolate stem cells using the aldefluor assay. Since then, ALDH isoform 1 (ALDH1) has been demonstrated to identify normal and malignant stem cells in several tissues (Cheung *et al.* 2007; Ginestier *et al.* 2007), including colon (Huang *et al.* 2009), and its high expression has been related to resistance to RT and chemotherapy (Chen *et al.* 2009; Oh *et al*. 2011). To the best of my knowledge, there is only one study (Kahlert *et al.* 2012) before ours that has specifically aimed at study the prognostic value of ALDH1 in rectal cancer, as the few others have analyzed colon and rectal tumors together (Lugli *et al.* 2010; Hessman *et al.* 2012; Vogler *et al.* 2012). No study has previously assessed ALDH1 protein expression specifically in relation to a material of rectal cancer patients treated with preoperative RT. In the study IV, we demonstrated ALDH1 expression to remain stable or to increase after RT in most of the cases. Importantly, positive expression predicted poor outcome in node-negative rectal cancer, possible due to resistance to chemotherapy.

In our study, ALDH1 protein expression above the cut-off value was mostly seen in the cell cytoplasm. In these cases, a relatively large number of ALDH1-positive cells could be detected similarly to earlier reports on ALDH1 (Hessmann *et al*. 2012) and other putative CSC markers (Horst *et al.* 2009). Our results thus support the theory of stem cell overpopulation during CRC progression (Huang *et al.* 2009) instead of the presence of only few percent of CSCs within a tumor (Ricci-Vitiani *et al.* 2007). Positive ALDH1 protein expression correlated with dysregulated pattern of nuclear β-catenin

expression which is considered as a feature of stem cell signaling activation (Reya *et al.* 2001). ALDH1-positive tumors more often expressed β-catenin in unregulated manner than ALDH1-negative tumors, which is not surprising considering the suggested involvement of both proteins in the maintenance of CRC stem cell population (Reya *et al.* 2001; Huang *et al.* 2009; Vermeulen *et al.* 2010). Earlier too, expression of β-catenin has been interrelated functionally with that of ALDH1 (Dillard and Lane, 2007). Aberrant activation of Wnt/ β-catenin signaling is considered as one of the earliest genetic abnormalities in colorectal carcinogenesis, and also ALDH1 expression has been demonstrated to increase already at early phases of carcinogenesis (Huang *et al.* 2009). Early-phase dysregulation of β-catenin and ALDH1 supports the implication of these proteins in CRC stemness, because CSCs are considered as the potential initiative cells in colorectal carcinogenesis (Reya *et al.* 2001).

ALDH1 may modulate stem cell proliferation, and its expression has been correlated with increased proliferative activity in several malignancies (Lohberger *et al*. 2012; Liang *et al.* 2012). Quite surprisingly, we found an inverse correlation between ALDH1 and Ki-67 expression in the biopsy samples taken before irradiation. Two explanations could be offered for our finding. First, even though CSCs are characterized by unlimited proliferative potential (Reya *et al.* 2001), they may be slowly cycling (Marcato *et al.* 2011). Ki-67 is not able to reflect the time required for the cell cycle, and accordingly, a strongly staining tumor may have a slow proliferation rate (Brown and Gatter, 1990). Second, ALDH1 is considered to affect cellular proliferation via its ability produce retinoic acid (Yoshida *et al.* 1992; Marchitti *et al.* 2008), which in turn is related to anti-proliferative actions (Tang and Gudas, 2011). In contrast to biopsy samples, no relation between ALDH1 and Ki-67 expression was seen in operative samples which could reflect the effect of RT on expression of these markers. Ki-67 as a pure proliferation marker was decreased after RT, whereas ALDH1 protein expression remained stable or increased in response to RT. However, as no correlation was seen neither in the operative samples of the surgery-only group, the actual association of ALDH1 with proliferation based on our material remains to be determined.

ALDH1 expression has been related to radioresistance in head and neck (Chen *et al.* 2009) and breast (Croker and Allan, 2012) cancer, agreeing with the idea that CSCs may repopulate malignant tumors during RT (Baumann *et al.* 2009). In accordance with this theory, most of the biopsy-operative sample pairs in our material showed either stabile or increased protein expression after RT, and irradiated patients more often presented with ALDH1-positive tumors compared to surgery-only patients. Our results may indicate a higher radioresistance for ALDH1-positive rectal cancer cells compared to ALDH1-negative cells. The underlying mechanisms may be the role of ALDH1 in cellular protection against oxidative stress, as well as the suggested slow growth rate of CSCs (Marcato *et al.* 2011). Nevertheless, no established conclusion can be constituted as ALDH1 expression in pre-irradiation biopsy samples did not relate to tumor regression grade after (C)RT.

ALDH1 may contribute to EMT (Chen *et al.* 2009) and increased invasive potential of cancer cells (Wang *et al.* 2012; Wakamatsu *et al.* 2012). EMT is a series of events where malignant epithelial cells are released from the surrounding tissues, enabling invasion and metastasis. Although positive ALDH1 expression correlated to aberrant expression of nuclear β-catenin, another potential activator of EMT (Yao *et al.* 2011), we did not find ALDH1 expression to differ between the invasive front and central parts of the tumor. Neither was ALDH1 protein expression correlated to lymph node status in our material, in contrast to reports in some other malignancies (Wang *et al.* 2012; Wakamatsu *et al.* 2012).

Poor disease outcome has been demonstrated in tumors showing high ALDH1 expression among others in breast (Ginestier *et al.* 2007), esophageal (Wang *et al.* 2012) and gastric (Wakamatsu *et al.* 2012) cancer. Regarding rectal cancer, Kahlert *et al.* (2012) did not find cytoplasmic ALDH1 expression to be of prognostic value, and the results from the studies including both colon and rectal tumors have not been unanimous (Lugli *et al.* 2010; Vogler *et al.* 2012), possibly reflecting the heterogeneity in antibodies and selected cut-off values for positive/high expression. We selected a cut-off value of 3% as a limit for positive expression, because ALDH1 expression may be seen in few percent of normal crypt cells, too (Huang *et al.* 2009). If lower limit was chosen, tumors with normal amount of ALDH1-expressing stem cells could have inaccurately been appointed as having high amount of ALDH1 expressing CSCs. We found high ALDH1 expression in operative samples to independently predict poor disease outcome (DFS and DSS) in node-negative population (stage I-II), specifically in those patients treated with postoperative adjuvant chemotherapy. Accordingly, in accordance with CSCs hypothesis (Reya *et al*. 2001), we suggest that tumor cells with high ALDH1 expression retain their capability to proliferate and disseminate into distant organs in spite of cytotoxic therapies. Our results are supported by others reporting decreased chemosensitivity in cancer cell lines and tumors rich in ALDH1 expression (Dylla *et al*. 2008; Oh *et al.* 2011; Steg *et al.* 2012). The resistance mechanisms may include slower growth rate of CSCs compared to bulk tumor cells, enhanced efflux of chemotherapeutic drugs, and detoxifying activity of ALDH1 as reviewed by Marcato *et al.* (2011).

The failure of ALDH1 expression to predict disease outcome in node-positive (III) cases could result from the strong prognostic value of lymph node metastasis itself. It might also relate to the RT-induced changes in tumor biology as stage III tumors are almost inevitably treated with preoperative RT. Indeed, when examining ALDH1-expression in pre-irradiation biopsies in relation to disease outcome, an apparent tendency towards shorter DFS and DSS was seen in ALDH1-positive cases, indicating that RT may interfere the interpretation of the prognostic value of ALDH1 when the analysis is based on post-irradiation samples. Accordingly, one could question the reliability of the results also in node-negative tumors, because a proportion of them was treated with preoperative RT. ALDH1 expression was, however, adjusted to preoperative treatment in the multivariate model, and remained as an independent prognostic factor both for DFS and DSS.

In summary, positive ALDH1 protein expression correlated with aberrant expression pattern of nuclear β-catenin, strengthening the role of ALDH1 in rectal cancer stem cell signaling. ALDH1 may indicate resistance to radio- and chemotherapy because its expression was very rarely decreased after RT, and because node-negative rectal cancer patients treated with postoperative chemotherapy had adverse outcome if they presented with high ALDH1-expressing tumors. This is an important finding, because postoperative treatment especially in stage II rectal cancer is not straightforward, and it is possible that patients with ALDH1-high tumors do not benefit from chemotherapy regimens that are traditionally utilized in adjuvant treatment of rectal cancer. However, this hypothesis should be studied in a homogenous patient material including larger number of stage II cases.

6.3. Tumor regression after long-course (chemo)radiotherapy

Preoperative chemoradiotherapy improves prognosis in locally advanced rectal cancer and enables tumor donwnstaging in up to 60% of cases (García-Aguilar *et al*. 2003). CRT induces histological changes in the tumor tissue, which are utilized in the analysis of tumor regression grade after treatment (Rödel *et al.* 2005; Bibeau *et al.* 2011). There is, however, no consensus of the method used in the evaluation of TRG after long-course CRT, although all scales base on analysis of the amount of residual tumor mass and fibrosis (Dworak *et al*. 1997; Rödel *et al.* 2005; Ryan *et al.* 2005). Moreover, the significance of other histological changes, such as colloid response and endocrine differentiation, remain indecisive (Bibeau *et al*. 2011). In some studies, three-point scales have been shown to result in superior inter-observer agreement as compared to five-point evaluation scales (Ryan *et al*. 2005). We used a three-point scale modified from Dworak (1997) and Rödel (2005) scales, with greater equivalence to the latter one. The rate of pathologic complete response in our material (5%) was lower than generally reported, which might reflect the rather large number of examined slides per tumor, but may also result from the relatively small size of the long-course (C)RT group. Similarly to earlier reports (Rödel *et al*. 2005; Ryan *et al.* 2005; Korkeila *et al.* 2011), excellent TRG related to improved DFS and DSS in univariate analysis. Instead, TRG did not remain as an independent prognosticator in the multivariate model. Of the three patients with pCR in our material, no one had died of rectal cancer.

Of the studied biomarkers, only high Ki-67 expression in operative samples correlated with adverse TRG, while securin had a parallel tendency. Predictive biomarkers should, however, be analyzed from the tissue samples taken prior to treatment, in this case from pre-irradiation biopsy samples. Using this criterion, none of the examined biomarkers succeeded to predict rectal cancer response to RT. It is possible that the small number of available biopsies, as well as their relatively small size, influenced the lack of correlations.

6.4. EGFR gene copy number (GCN) (V)

Cetuximab and panitumumab are EGFR-targeted monoclonal antibodies with efficacy in treatment of *KRAS* WT metastatic CRC (Lièvre *et al.* 2006; Amado *et al.* 2008). However, even among *KRAS* WT population, clinical benefit is achieved only in less than 50% of patients depending on the line of treatment, the median response rate being approximately 35% in chemorefractory disease (Chang *et al.* 2009). Accordingly, additional predictive factors are urgently demanded. Our group recently demonstrated that a heterogeneous increase in *EGFR* GCN is a highly significant predictor of responsiveness to EGFR-targeted antibodies in terms of improved clinical benefit rate, PFS and OS (Ålgars *et al.* 2011). In the study V, we validated this previous result with an independent patient cohort, now concentrating on chemorefractory patient population. The positive correlation between *EGFR* GCN and cancer cell death caused by EGFR-targeted antibodies was also confirmed with CRC cell lines. In addition, we showed that the *EGFR* GCN is highly heterogeneous in CRC, and that the average GCN without selection of the cells with the highest copy number fails to predict benefit from EGFR-targeted antibodies.

KRAS mutation testing is the only predictive biomarker in clinical use for the treatment of CRC, and is highly specific for negative response to EGFR-targeted antibodies (Lièvre *et al.* 2006; Amado *et al*. 2008). However, it lacks sufficient sensitivity indicating that a substantial proportion of *KRAS* WT patients do not benefit from treatment with cetuximab or panitumumab (Chang *et al.* 2009). Our group recently demonstrated that *EGFR* GCN, as analyzed with a relatively novel silver-enhanced *in situ* hybridization (SISH) technique, was useful in predicting benefit from EGFR-targeted antibodies in the treatment of metastatic CRC (Ålgars *et al.* 2011). Increased *EGFR* GCN (≥4) distinguished *KRAS* WT patients who most likely benefited from treatment with EGFR-targeted antibody (Ålgars *et al.* 2011). Parallel findings have previously been presented using other *in situ* hybridization-based techniques (Moroni *et al.* 2005; Scartozzi *et al.* 2009), but technical difficulties, heterogeneity between the scoring systems, and a relatively poor reproducibility have impeded their clinical usefulness (Yang *et al.* 2012). SISH is a fully automated technique using silver chromogens for enzymatic labelling. Compared to FISH, it is more rapid, has improved signal stability, and enables *EGFR* gene and chromosome analysis using a conventional bright field microscopy (Dietel *et al.* 2007). Thus, molecular diagnostics within the context of tissue morphology is possible, and SISH-based evaluation of *HER2* amplification is already in clinical use for breast and gastric cancer. In the present study, we were able to repeat our previous results (Ålgars *et al.* 2011) with an individual patient cohort. Similarly to original cohort, patients in the validation cohort presenting with increased GCN-tumors (≥4) had improved clinical benefit rate, PFS, and OS compared to patients with tumors showing under four gene copies. To minimize the confounding effect of chemotherapy treatment on response evaluation of EGFR-targeted antibodies, *EGFR* GCN was separately analyzed in chemorefractory population. The results were highly similar to those obtained in

analysis of the whole patient cohort, supporting the predictive value of *EGFR* GCN on the treatment of metastatic CRC. Our findings were further substantiated by the cell line studies, in which the *EGFR* GCN associated positively with EGFR protein expression in Western blot analysis, and the *KRAS* WT cells with increased *EGFR* GCN were the most susceptible to cetuximab- and panitumumab-induced cell death. Interestingly, *KRAS* mutant cells with increased *EGFR* GCN appeared to be more sensitive for EGFR-targeted antibodies than the *KRAS* WT cells with disomic *EGFR* GCN, thus underlining the importance of both *EGFR* GCN and *KRAS* status in CRC response to EGFR-targeted therapies.

The positive predictive value of increased *EGFR* GCN *in vivo* was stronger for benefit from cetuximab than from panitumumab, whereas no differences were seen *in vitro*. PFS was improved in patients with increased GCN tumors regardless of the administered antibody, but the clinical benefit rate and OS were statistically significantly improved only in patients treated with cetuximab. Although the lack of statistical significance may simply reflect the smaller number of patients treated with panitumumab, there might be additional underlying mechanisms. In spite of their similar mechanisms of action, i.e. binding to and inhibition of EGFR, cetuximab and panitumumab are not identical. The former is a chimeric (mouse/human) immunoglobulin (Ig) G1 antibody, while the latter is a fully humanized IgG2 antibody (Kasper *et al.* 2012). Panitumumab has been associated with higher affinity to EGFR than cetuximab *in vitro* (Saxena *et al.* 2011), but cetuximab may possess superior therapeutic activity *in vivo*, possibly because it is capable of inducing antibody-dependent cytotoxicity (Kasper *et al.* 2012). This effect is more pronounced in *KRAS* WT cells and *in vivo* circumstances than it is in *KRAS* mutant cells and *in vitro* circumstances (Kasper *et al.* 2012), potentially explaining why the cytotoxic effect of cetuximab and panitumumab were rather similar in cell lines in spite of their differences in clinical CRC cases.

In the era of personalized medicine, intratumor heterogeneity has attracted increasing interest. Single tumor may present with distinct gene expression profiles and biologic properties, and this diversity may aid in selecting the clones that have the most advantageous properties for tumor progression (Marusyk *et al*. 2012). This may complicate the validation of prognostic and predictive biomarkers. Moreover, the use of primary tumors in treatment decisions of metastatic disease may be questioned, because mutational status of primary and metastatic tumor has been demonstrated to be dissimilar in some cases (Baldus *et al.* 2010), and adjuvant treatment of the primary tumor may even further influence the mutational spectrum (Niikura *et al.* 2012; Bai *et al.* 2012). In accordance with others (Moroni *et al.* 2005), we demonstrated that *EGFR* GCN increase is heterogeneous in CRC. With regard to other cancers, intratumor heterogeneity has been demonstrated in terms of *EGFR* and *HER2* amplification among others in glioblastoma (Snuderl *et al.* 2011) and gastric cancer (Kim *et al*. 2011). In CRC, a true amplification of the *EGFR* gene is a rare event, and the increased GCN usually results from chromosome 7 polysomy (Yang *et al.* 2012).

Heterogeneity has been suggested to be an important player in resistance to targeted cancer therapies, because scoring of the potentially predictive molecules has traditionally been based only on the dominant phenotype (Marusyk *et al*. 2012). Agreeing with this theory, we showed that only the method extracting cells with the highest *EGFR* GCN associated significantly with clinical benefit from cetuximab and panitumumab, whereas scoring the GCN in randomly selected cells failed to be of similar value. Thus, it seems conceivable that the relatively small population of cells with increased *EGFR* GCN possess distinct biological properties enabling determination of the fate of cancer cells within the larger tumor bulk. The reasons for this may only be speculated, but could relate to suggested stem cell properties of epidermal growth factor signaling pathway (Feng *et al.* 2012). Moreover, under inhibition with EGFR-targeted antibody, *EGFR* GCN low cells might be more susceptible to compensatory activation of other receptor tyrosine kinases and their downstream signaling pathways.

As a conclusion, *EGFR* GCN increase is heterogeneous in CRC, and disregarding this heterogeneity may have resulted in the previously reported reproducibility problems using other techniques. The use of SISH-based *EGFR* GCN analysis as a predictive tool for EGFR-targeted treatment response is promising as we were able to validate our previous results in an individual patient cohort, and in a combined group of chemorefractory patients. The predictive value may differ among cetuximab and panitumumab-treated patients, which might reflect the differences of these antibodies.

7. SUMMARY AND CONCLUSIONS

There is a substantial lack of clinically useful prognostic and predictive biomarkers in colorectal cancer in spite of large number of studies in this field. Based on the studies included in this thesis, following conclusions can be made:

1) IIA PLA2 expression is decreased in CRC compared to adenomas, potentially indicating a protective role for IIA PLA2 against development of invasive carcinoma. IIA PLA2 was not prognostic factor for disease outcome (*unpublished data*).

2) Securin and Ki-67 protein expression in rectal cancer decrease after exposure to (chemo)radiotherapy, but their pre-irradiation expression levels are not predictive for TRG. High securin protein expression after long-course (C)RT seems to be an independent prognostic factor for poor disease-specific survival.

3) The localization of membranous CD44v6 protein expression is more important than its total amount of expression in rectal cancer. The lack of this protein in invasive front may associate with aggressive phenotype and shortened disease-free survival, but is not an independent adverse prognostic factor in rectal cancer.

4) ALDH1 protein expression remains stable or shows an increase in most of the cases after preoperative (C)RT, but its predictive usefulness for rectal cancer response to this treatment modality remains unresolved. In node-negative rectal cancer, increased ALDH1 protein expression may indicate adverse disease-free and disease-specific survival, and might be predictive for chemotherapy resistance.

5) *EGFR* GCN increase is highly heterogeneous in CRC. The results from our validation cohort strengthen the view that calculating *EGFR* GCN from the cells with the highest copy number is an encouraging predictive tool for benefit from EGFR-targeted antibodies. It is possible that the predictive power is somewhat different for cetuximab and panitumumab.

Several biomarkers are promising in predicting CRC outcome, but identification of prognostic marker for wider clinical use is challenging. This may reflect the heterogeneity of CRC, supporting the need for personalized medicine. Increased *EGFR* GCN is a very promising biomarker for identification of the patients who are likely to benefit from cetuximab and panitumumab.

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9. REFERENCES

Adjuvant therapy for patients with colon and rectum cancer. *Consens Statement* 1990; 8: 1-25

Al-Sohaily S, Biankin A, Leong R, Kohonen-Corish M and Warusavitarne J. Molecular pathways in colorectal cancer. *J Gastroenterol Hepatol* 2012; 27: 1423-31

Alberts SR, Sargent DJ, Nair S, Mahoney MR, Mooney M, Thibodeau SN, Smyrk TC, Sinicrope FA, Chan E, Gill S, Kahlenberg MS, Shields AF, Quesenberry JT, Webb TA, Farr GH, Pockaj BA, Grothey A and Goldberg RM. Effect of oxaliplatin, fluorouracil, and leucovorin with or without cetuximab on survival among patients with resected stage III colon cancer: a randomized trial. *JAMA* 2012; 307: 1383-93

Alhopuro P, Sammalkorpi H, Niittymäki I, Biström M, Raitila A, Saharinen J, Nousiainen K, Lehtonen HJ, Heliövaara E, Puhakka J, Tuupanen S, Sousa S, Seruca R, Ferreira AM, Hofstra RM, Mecklin JP, Järvinen H, Ristimäki A, Orntoft TF, Hautaniemi S, Arango D, Karhu A and Aaltonen LA. Candidate driver genes in microsatellite-unstable colorectal cancer. *Int J Cancer* 2012; 130: 1558-66

Allegra CJ, Paik S, Colangelo LH, Parr AL, Kirsch I, Kim G, Klein P, Johnston PG, Wolmark N and Wieand HS. Prognostic value of thymidylate synthase, Ki-67, and p53 in patients with Dukes' B and C colon cancer: a National Cancer Institute-National Surgical Adjuvant Breast and Bowel Project collaborative study. *J Clin Oncol* 2003; 21: 241-50

Allegra CJ, Yothers G, O'Connell MJ, Sharif S, Petrelli NJ, Colangelo LH, Atkins JN, Seay TE, Fehrenbacher L, Goldberg RM, O'Reilly S, Chu L, Azar CA, Lopa S and Wolmark N. Phase III trial assessing bevacizumab in stages II and III carcinoma of the colon: results of NSABP protocol C-08. *J Clin Oncol* 2011; 29: 11-6

American Joint Committee on Cancer (AJCC). Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A III (eds). Chapter 14: Colon and Rectum *in* Cancer Staging Handbook, 7th edition, 2010, 183 - 91, Springer**,** New York, NY

Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, Juan T, Sikorski R, Suggs S, Radinsky R, Patterson SD and Chang DD. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol* 2008; 26: 1626-34

Anaya DA, Chang GJ and Rodriguez-Bigas MA. Extracolonic manifestations of hereditary colorectal cancer syndromes. *Clin Colon Rectal Surg* 2008; 21: 263-72

André T, Boni C, Mounedji-Boudiaf L, Navarro M, Tabernero J, Hickish T, Topham C, Zaninelli M, Clingan P, Bridgewater J, Tabah-Fisch I, de Gramont A; Multicenter International Study of Oxaliplatin/5-Fluorouracil/Leucovorin in the Adjuvant Treatment of Colon Cancer (MOSAIC) Investigators. Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. *N Engl J Med* 2004; 350: 2343-51

André T, Boni C, Navarro M, Tabernero J, Hickish T, Topham C, Bonetti A, Clingan P, Bridgewater J, Rivera F and de Gramont A. Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial. *J Clin Oncol* 2009; 27: 3109-16

Atkin WS, Edwards R, Kralj-Hans I, Wooldrage K, Hart AR, Northover JM, Parkin DM, Wardle J, Duffy SW, Cuzick J and Investigators UFST. Once-only flexible sigmoidoscopy screening in prevention of colorectal cancer: a multicentre randomised controlled trial. *Lancet* 2010; 375: 1624-33

Aune D, Chan DS, Lau R, Vieira R, Greenwood DC, Kampman E and Norat T. Dietary fibre, whole grains, and risk of colorectal cancer: systematic review and dose-response meta-analysis of prospective studies. *BMJ* 2011; 343: d6617

Bai H, Wang Z, Chen K, Zhao J, Lee JJ, Wang S, Zhou Q, Zhuo M, Mao L, An T, Duan J, Yang L, Wu M, Liang Z, Wang Y, Kang X and Wang J. Influence of chemotherapy on EGFR mutation status among patients with non-small-cell lung cancer. *J Clin Oncol* 2012; 30: 3077-83

Baldus SE, Schaefer KL, Engers R, Hartleb D, Stoecklein NH and Gabbert HE. Prevalence and heterogeneity of KRAS, BRAF, and PIK3CA mutations in primary colorectal adenocarcinomas and their corresponding metastases. *Clin Cancer Res* 2010; 16: 790-9

Balmaña J, Castells A, Cervantes A and Group EGW. Familial colorectal cancer risk: ESMO Clinical Practice Guidelines. *Ann Oncol* 2010; 21 Suppl 5: v78-81

Bardelli A and Siena S. Molecular mechanisms of resistance to cetuximab and panitumumab in colorectal cancer. *J Clin Oncol* 2010; 28: 1254-61

Baumann M, Krause M, Thames H, Trott K and Zips D. Cancer stem cells and radiotherapy. *Int J Radiat Biol* 2009; 85: 391-402

Baxter NN, Tepper JE, Durham SB, Rothenberger DA and Virnig BA. Increased risk of rectal cancer

after prostate radiation: a population-based study. *Gastroenterology* 2005; 128: 819-24

Baxter NN, Virnig DJ, Rothenberger DA, Morris AM, Jessurun J and Virnig BA. Lymph node evaluation in colorectal cancer patients: a population-based study. *J Natl Cancer Inst* 2005; 97: 219-25

Belinsky GS, Rajan TV, Saria EA, Giardina C and Rosenberg DW. Expression of secretory phospholipase A2 in colon tumor cells potentiates tumor growth. *Mol Carcinog* 2007; 46: 106-16

Bendardaf R, Algars A, Elzagheid A, Korkeila E, Ristamäki R, Lamlum H, Collan Y, Syrjänen K and Pyrhönen S. Comparison of CD44 expression in primary tumours and metastases of colorectal cancer. *Oncol Rep* 2006; 16: 741-6

Bendardaf R, Elzagheid A, Lamlum H, Ristamäki R, Collan Y and Pyrhönen S. E-cadherin, CD44s and CD44v6 correlate with tumour differentiation in colorectal cancer. *Oncol Rep* 2005; 13: 831-5

Bendardaf R, Lamlum H, Ristamäki R and Pyrhönen S. CD44 variant 6 expression predicts response to treatment in advanced colorectal cancer. *Oncol Rep* 2004; 11: 41-5

Bennett KL, Jackson DG, Simon JC, Tanczos E, Peach R, Modrell B, Stamenkovic I, Plowman G and Aruffo A. CD44 isoforms containing exon V3 are responsible for the presentation of heparin-binding growth factor. *J Cell Biol* 1995; 128: 687-98

Bernal JA, Luna R, Espina A, Lázaro I, Ramos-Morales F, Romero F, Arias C, Silva A, Tortolero M and Pintor-Toro JA. Human securin interacts with p53 and modulates p53-mediated transcriptional activity and apoptosis. *Nat Genet* 2002; 32: 306-11

Bernal JA, Roche M, Méndez-Vidal C, Espina A, Tortolero M and Pintor-Toro JA. Proliferative potential after DNA damage and non-homologous end joining are affected by loss of securin. *Cell Death Differ* 2008; 15: 202-12

Bernstein TE, Endreseth BH, Romundstad P, Wibe A; Norwegian Colorectal Cancer Registry. Improved local control of rectal cancer reduces distant metastases. *Colorectal Dis* 2012; 14: e668-e678

Bertolini F, Bengala C, Losi L, Pagano M, Iachetta F, Dealis C, Jovic G, Depenni R, Zironi S, Falchi AM, Luppi G and Conte PF. Prognostic and predictive value of baseline and posttreatment molecular marker expression in locally advanced rectal cancer treated with neoadjuvant chemoradiotherapy. *Int J Radiat Oncol Biol Phys* 2007; 68: 1455-61

Betge J, Pollheimer MJ, Lindtner RA, Kornprat P, Schlemmer A, Rehak P, Vieth M, Hoefler G and Langner C. Intramural and extramural vascular invasion in colorectal cancer: prognostic significance and quality of pathology reporting. *Cancer* 2012; 118: 628-38

Bibeau F, Rullier A, Jourdan MF, Frugier H, Palasse J, Leaha C, Gudin de Vallerin A, Rivière B, Bodin X, Perrault V, Cantos C, Lavaill R, Boissière-Michot F, Azria D, Colombo PE, Rouanet P, Rullier E, Panis Y and Guedj N. Locally advanced rectal cancer management: which role for the pathologist in 2011?. *Ann Pathol* 2011; 31: 433-41

Bird RP. Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. *Cancer Lett* 1987; 37: 147-51

Birgisson H, Påhlman L, Gunnarsson U and Glimelius B. Late adverse effects of radiation therapy for rectal cancer - a systematic overview. *Acta Oncol* 2007; 46: 504-16

Bosetti C, Bravi F, Negri E and La Vecchia C. Oral contraceptives and colorectal cancer risk: a systematic review and meta-analysis. *Hum Reprod Update* 2009; 15: 489-98

Bosset JF, Calais G, Mineur L, Maingon P, Radosevic-Jelic L, Daban A, Bardet E, Beny A, Briffaux A and Collette L. Enhanced tumorocidal effect of chemotherapy with preoperative radiotherapy for rectal cancer: preliminary results--EORTC 22921. *J Clin Oncol* 2005; 23: 5620-7

Brabletz T, Jung A, Hermann K, Günther K, Hohenberger W and Kirchner T. Nuclear overexpression of the oncoprotein beta-catenin in colorectal cancer is localized predominantly at the invasion front. *Pathol Res Pract* 1998; 194: 701-4

Brown DC and Gatter KC. Monoclonal antibody Ki-67: its use in histopathology. *Histopathology* 1990; 17: 489-503

Brown DC and Gatter KC. Ki67 protein: the immaculate deception? *Histopathology* 2002; 40: 2-11

Brown G, Radcliffe AG, Newcombe RG, Dallimore NS, Bourne MW and Williams GT. Preoperative assessment of prognostic factors in rectal cancer using high-resolution magnetic resonance imaging. *Br J Surg* 2003a; 90: 355-64

Brown G, Richards CJ, Bourne MW, Newcombe RG, Radcliffe AG, Dallimore NS and Williams GT. Morphologic predictors of lymph node status in rectal cancer with use of high-spatial-resolution MR imaging with histopathologic comparison. *Radiology* 2003b; 227: 371-7

Buhmeida A, Bendardaf R, Hilska M, Laine J, Collan Y, Laato M, Syrjänen K and Pyrhönen S. PLA2 (group IIA phospholipase A2) as a prognostic determinant in stage II colorectal carcinoma. *Ann Oncol* 2009; 20: 1230-5

Bujko K, Nowacki MP, Nasierowska-Guttmejer A, Michalski W, Bebenek M and Kryj M. Long-term results of a randomized trial comparing preoperative short-course radiotherapy with preoperative

conventionally fractionated chemoradiation for rectal cancer. *Br J Surg* 2006; 93: 1215-23

Bullwinkel J, Baron-Lühr B, Lüdemann A, Wohlenberg C, Gerdes J and Scholzen T. Ki-67 protein is associated with ribosomal RNA transcription in quiescent and proliferating cells. *J Cell Physiol* 2006; 206: 624-35

Cao Y, Pearman AT, Zimmerman GA, McIntyre TM and Prescott SM. Intracellular unesterified arachidonic acid signals apoptosis. *Proc Natl Acad Sci U S A* 2000; 97: 11280-5

Carpenter G, King L and Cohen S. Epidermal growth factor stimulates phosphorylation in membrane preparations in vitro. *Nature* 1978; 276: 409-10

Chan DS, Lau R, Aune D, Vieira R, Greenwood DC, Kampman E and Norat T. Red and processed meat and colorectal cancer incidence: meta-analysis of prospective studies. *PLoS One* 2011; 6: e20456

Chang DZ, Kumar V, Ma Y, Li K and Kopetz S. Individualized therapies in colorectal cancer: KRAS as a marker for response to EGFR-targeted therapy. *J Hematol Oncol* 2009; 2: 18

Chari RS, Tyler DS, Anscher MS, Russell L, Clary BM, Hathorn J and Seigler HF. Preoperative radiation and chemotherapy in the treatment of adenocarcinoma of the rectum. *Ann Surg* 1995; 221: 778-86; discussion 786-7

Chen WS, Yu YC, Lee YJ, Chen JH, Hsu HY and Chiu SJ. Depletion of securin induces senescence after irradiation and enhances radiosensitivity in human cancer cells regardless of functional p53 expression. *Int J Radiat Oncol Biol Phys* 2010; 77: 566-74

Chen Y, Orlicky DJ, Matsumoto A, Singh S, Thompson DC and Vasiliou V. Aldehyde dehydrogenase 1B1 (ALDH1B1) is a potential biomarker for human colon cancer. *Biochem Biophys Res Commun* 2011; 405: 173-9

Chen YC, Chen YW, Hsu HS, Tseng LM, Huang PI, Lu KH, Chen DT, Tai LK, Yung MC, Chang SC, Ku HH, Chiou SH and Lo WL. Aldehyde dehydrogenase 1 is a putative marker for cancer stem cells in head and neck squamous cancer. *Biochem Biophys Res Commun* 2009; 385: 307-13

Cheung AM, Wan TS, Leung JC, Chan LY, Huang H, Kwong YL, Liang R and Leung AY. Aldehyde dehydrogenase activity in leukemic blasts defines a subgroup of acute myeloid leukemia with adverse prognosis and superior NOD/SCID engrafting potential. *Leukemia* 2007; 21: 1423-30

Chiu SJ, Hsu TS and Chao JI. Opposing securin and p53 protein expression in the oxaliplatin-induced cytotoxicity of human colorectal cancer cells. *Toxicol Lett* 2006; 167: 122-30

Chung KY, Shia J, Kemeny NE, Shah M, Schwartz GK, Tse A, Hamilton A, Pan D, Schrag D, Schwartz L, Klimstra DS, Fridman D, Kelsen DP and Saltz LB. Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. *J Clin Oncol* 2005; 23: 1803-10

Cirocchi R, Trastulli S, Abraha I, Vettoretto N, Boselli C, Montedori A, Parisi A, Noya G and Platell C. Non-resection versus resection for an asymptomatic primary tumour in patients with unresectable stage IV colorectal cancer. Cochrane *Database Syst Rev* 2012; 8: CD008997

Cohen RB. Epidermal growth factor receptor as a therapeutic target in colorectal cancer. *Clin Colorectal Cancer* 2003; 2: 246-51

Cole BF, Logan RF, Halabi S, Benamouzig R, Sandler RS, Grainge MJ, Chaussade S and Baron JA. Aspirin for the chemoprevention of colorectal adenomas: meta-analysis of the randomized trials. *J Natl Cancer Inst* 2009; 101: 256-66

Collette L, Bosset JF, den Dulk M, Nguyen F, Mineur L, Maingon P, Radosevic-Jelic L, Piérart M, Calais G and Group EOfRaToCRO. Patients with curative resection of cT3-4 rectal cancer after preoperative radiotherapy or radiochemotherapy: does anybody benefit from adjuvant fluorouracil-based chemotherapy? A trial of the European Organisation for Research and Treatment of Cancer Radiation Oncology Group. *J Clin Oncol* 2007; 25: 4379-86

Collins D, Hogan AM and Winter DC. Microbial and viral pathogens in colorectal cancer. *Lancet Oncol* 2011; 12: 504-12

Compton CC. Pathologic prognostic factors in the recurrence of rectal cancer. *Clin Colorectal Cancer* 2002; 2: 149-60

Cook NR, Lee IM, Gaziano JM, Gordon D, Ridker PM, Manson JE, Hennekens CH and Buring JE. Low-dose aspirin in the primary prevention of cancer: the Women's Health Study: a randomized controlled trial. *JAMA* 2005; 294: 47-55

Cooper HS. Pathologic issues in the treatment of endoscopically removed malignant colorectal polyps. *J Natl Compr Canc Netw* 2007; 5: 991-6

Coppola D, Hyacinthe M, Fu L, Cantor AB, Karl R, Marcet J, Cooper DL, Nicosia SV and Cooper HS. CD44V6 expression in human colorectal carcinoma. *Hum Pathol* 1998; 29: 627-35

Cormier RT, Hong KH, Halberg RB, Hawkins TL, Richardson P, Mulherkar R, Dove WF and Lander ES. Secretory phospholipase Pla2g2a confers resistance to intestinal tumorigenesis. *Nat Genet* 1997; 17: 88-91

Cosimelli M, Mancini R, Carpanese L, Sciuto R, Pizzi G, Pattaro G and Maini CL. Integration of radioembolisation into multimodal treatment of

liver-dominant metastatic colorectal cancer. *Expert Opin Ther Targets* 2012; 16 Suppl 2: S11-6

Croker AK and Allan AL. Inhibition of aldehyde dehydrogenase (ALDH) activity reduces chemotherapy and radiation resistance of stem-like ALDHhiCD44$^+$ human breast cancer cells. *Breast Cancer Res Treat* 2012; 133: 75-87

Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, Bets D, Mueser M, Harstrick A, Verslype C, Chau I and Van Cutsem E. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N Engl J Med* 2004; 351: 337-45

Dahl O, Fluge Ø, Carlsen E, Wiig JN, Myrvold HE, Vonen B, Podhorny N, Bjerkeset O, Eide TJ, Halvorsen TB, Tveit KM and Group NGC. Final results of a randomised phase III study on adjuvant chemotherapy with 5 FU and levamisol in colon and rectum cancer stage II and III by the Norwegian Gastrointestinal Cancer Group. *Acta Oncol* 2009; 48: 368-76

Dalchau R, Kirkley J and Fabre JW. Monoclonal antibody to a human leukocyte-specific membrane glycoprotein probably homologous to the leukocyte-common (L-C) antigen of the rat. *Eur J Immunol* 1980; 10: 737-44

Dalerba P, Dylla SJ, Park IK, Liu R, Wang X, Cho RW, Hoey T, Gurney A, Huang EH, Simeone DM, Shelton AA, Parmiani G, Castelli C and Clarke MF. Phenotypic characterization of human colorectal cancer stem cells. *Proc Natl Acad Sci U S A* 2007; 104: 10158-63

Davies RL, Grosse VA, Kucherlapati R and Bothwell M. Genetic analysis of epidermal growth factor action: assignment of human epidermal growth factor receptor gene to chromosome 7. *Proc Natl Acad Sci U S A* 1980; 77: 4188-92

de Gramont A, Figer A, Seymour M, Homerin M, Hmissi A, Cassidy J, Boni C, Cortes-Funes H, Cervantes A, Freyer G, Papamichael D, Le Bail N, Louvet C, Hendler D, de Braud F, Wilson C, Morvan F and Bonetti A. Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer. *J Clin Oncol* 2000; 18: 2938-47

de Jong MC, Pramana J, van der Wal JE, Lacko M, Peutz-Kootstra CJ, de Jong JM, Takes RP, Kaanders JH, van der Laan BF, Wachters J, Jansen JC, Rasch CR, van Velthuysen ML, Grénman R, Hoebers FJ, Schuuring E, van den Brekel MW and Begg AC. CD44 expression predicts local recurrence after radiotherapy in larynx cancer. *Clin Cancer Res* 2010; 16: 5329-38

de la Chapelle A. Genetic predisposition to colorectal cancer. *Nat Rev Cancer* 2004; 4: 769-80

De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilas G, Kalogeras KT, Kotoula V, Papamichael D, Laurent-Puig P, Penault-Llorca F, Rougier P, Vincenzi B, Santini D, Tonini G, Cappuzzo F, Frattini M, Molinari F, Saletti P, De Dosso S, Martini M, Bardelli A, Siena S, Sartore-Bianchi A, Tabernero J, Macarulla T, Di Fiore F, Gangloff AO, Ciardiello F, Pfeiffer P, Qvortrup C, Hansen TP, Van Cutsem E, Piessevaux H, Lambrechts D, Delorenzi M, Tejpar S. Effects of KRAS, BRAF, NRAS, and PI3KCA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. *Lancet Oncol* 2010a; 11: 753-62

De Roock W, Jonker DJ, Di Nicolantonio F, Sartore-Bianchi A, Tu D, Siena S, Lamba S, Arena S, Frattini M, Piessevaux H, Van Cutsem E, O'Callaghan CJ, Khambata-Ford S, Zalcberg JR, Simes J, Karapetis CS, Bardelli A and Tejpar S. Association of KRAS p.G13D mutation with outcome in patients with chemotherapy-refractory metastatic colorectal cancer treated with cetuximab. *JAMA* 2010b; 304: 1812-20

Debucquoy A, Goethals L, Libbrecht L, Perneel C, Geboes K, Ectors N, McBride WH and Haustermans K. Molecular and clinico-pathological markers in rectal cancer: a tissue micro-array study. *Int J Colorectal Dis* 2009; 24: 129-38

Denekamp J. Cell kinetics and radiation biology. Int *J Radiat Biol Relat Stud Phys Chem Med* 1986; 49: 357-80

Deng S, Yang X, Lassus H, Liang S, Kaur S, Ye Q, Li C, Wang LP, Roby KF, Orsulic S, Connolly DC, Zhang Y, Montone K, Bützow R, Coukos G and Zhang L. Distinct expression levels and patterns of stem cell marker, aldehyde dehydrogenase isoform 1 (ALDH1), in human epithelial cancers. *PLoS One* 2010; 5: e10277

Dietel M, Ellis IO, Höfler H, Kreipe H, Moch H, Dankof A, Kölble K and Kristiansen G. Comparison of automated silver enhanced in situ hybridisation (SISH) and fluorescence ISH (FISH) for the validation of HER2 gene status in breast carcinoma according to the guidelines of the American Society of Clinical Oncology and the College of American Pathologists. *Virchows Arch* 2007; 451: 19-25

Dillard AC and Lane MA. Retinol decreases beta-catenin protein levels in retinoic acid-resistant colon cancer cell lines. *Mol Carcinog* 2007; 46: 315-29

Domínguez A, Ramos-Morales F, Romero F, Rios RM, Dreyfus F, Tortolero M and Pintor-Toro JA. hpttg, a human homologue of rat pttg, is overexpressed in hematopoietic neoplasms. Evidence for a transcriptional activation function of hPTTG. *Oncogene* 1998; 17: 2187-93

Douillard JY, Siena S, Cassidy J, Tabernero J, Burkes R, Barugel M, Humblet Y, Bodoky G, Cunningham D, Jassem J, Rivera F, Kocákova I, Ruff P, Błasińska-Morawiec M, Šmakal M, Canon JL, Rother M, Oliner KS, Wolf M and Gansert J. Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: the PRIME study. *J Clin Oncol* 2010; 28: 4697-705

Dworak O, Keilholz L and Hoffmann A. Pathological features of rectal cancer after preoperative radiochemotherapy. *Int J Colorectal Dis* 1997; 12: 19-23

Dylla SJ, Beviglia L, Park IK, Chartier C, Raval J, Ngan L, Pickell K, Aguilar J, Lazetic S, Smith-Berdan S, Clarke MF, Hoey T, Lewicki J and Gurney AL. Colorectal cancer stem cells are enriched in xenogeneic tumors following chemotherapy. *PLoS One* 2008; 3: e2428

Eaden JA, Abrams KR and Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut* 2001; 48: 526-35

Eche N, Pichon MF, Quillien V, Gory-Delabaere G, Riedinger JM, Basuyau JP, Daver A, Buecher B, Conroy T, Dieu L, Bidart JM, Deneux L; Groupe de travail Nicole Eche (coordonnateur), oncobiologiste. Standards, options and recommendations for tumor markers in colorectal cancer. *Bull Cancer* 2001; 88: 1177-206

Edhemović I, Snoj M, Kljun A and Golouh R. Immunohistochemical localization of group II phospholipase A2 in the tumours and mucosa of the colon and rectum. *Eur J Surg Oncol* 2001; 27: 545-8

Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, Rubinstein L, Shankar L, Dodd L, Kaplan R, Lacombe D and Verweij J. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer* 2009; 45: 228-47

Engels B, Gevaert T, Sermeus A and De Ridder M. Current status of intensified neo-adjuvant systemic therapy in locally advanced rectal cancer. *Front Oncol* 2012; 2: 47

Faleiro-Rodrigues C and Lopes C. E-cadherin, CD44 and CD44v6 in squamous intraepithelial lesions and invasive carcinomas of the uterine cervix: an immunohistochemical study. *Pathobiology* 2004; 71: 329-36

Fearon ER and Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990; 61: 759-67

Fedirko V, Tramacere I, Bagnardi V, Rota M, Scotti L, Islami F, Negri E, Straif K, Romieu I, La Vecchia C, Boffetta P and Jenab M. Alcohol drinking and colorectal cancer risk: an overall and dose-response meta-analysis of published studies. *Ann Oncol* 2011; 22: 1958-72

Feng Y, Dai X, Li X, Wang H, Liu J, Zhang J, Du Y and Xia L. EGF signalling pathway regulates colon cancer stem cell proliferation and apoptosis. *Cell Prolif* 2012; 45: 413-9

Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010; 127: 2893-917

Fijneman RJ and Cormier RT. The roles of sPLA2-IIA (Pla2g2a) in cancer of the small and large intestine. *Front Biosci* 2008; 13: 4144-74

Filippella M, Galland F, Kujas M, Young J, Faggiano A, Lombardi G, Colao A, Meduri G and Chanson P. Pituitary tumour transforming gene (PTTG) expression correlates with the proliferative activity and recurrence status of pituitary adenomas: a clinical and immunohistochemical study. *Clin Endocrinol (Oxf)* 2006; 65: 536-43

Fiorica F, Cartei F, Licata A, Enea M, Ursino S, Colosimo C and Cammà C. Can chemotherapy concomitantly delivered with radiotherapy improve survival of patients with resectable rectal cancer? A meta-analysis of literature data. *Cancer Treat Rev* 2010; 36: 539-49

Fisher B, Wolmark N, Rockette H, Redmond C, Deutsch M, Wickerham DL, Fisher ER, Caplan R, Jones J and Lerner H. Postoperative adjuvant chemotherapy or radiation therapy for rectal cancer: results from NSABP protocol R-01. *J Natl Cancer Inst* 1988; 80: 21-9

Folkesson J, Birgisson H, Pahlman L, Cedermark B, Glimelius B and Gunnarsson U. Swedish Rectal Cancer Trial: long lasting benefits from radiotherapy on survival and local recurrence rate. *J Clin Oncol* 2005; 23: 5644-50

Fonatsch C, Duchrow M, Rieder H, Schlüter C and Gerdes J. Assignment of the human Ki-67 gene (MK167) to 10q25-qter. *Genomics* 1991; 11: 476-7

Galandiuk S, Wieand HS, Moertel CG, Cha SS, Fitzgibbons RJ, Pemberton JH and Wolff BG. Patterns of recurrence after curative resection of carcinoma of the colon and rectum. *Surg Gynecol Obstet* 1992; 174: 27-32

Ganesan K, Ivanova T, Wu Y, Rajasegaran V, Wu J, Lee MH, Yu K, Rha SY, Chung HC, Ylstra B, Meijer G, Lian KO, Grabsch H and Tan P. Inhibition of gastric cancer invasion and metastasis by PLA2G2A, a novel beta-catenin/TCF target gene. *Cancer Res* 2008; 68: 4277-86

Garcia-Albeniz X and Chan AT. Aspirin for the prevention of colorectal cancer. *Best Pract Res Clin Gastroenterol* 2011; 25: 461-72

García-Aguilar J, Hernandez de Anda E, Sirivongs P, Lee SH, Madoff RD and Rothenberger DA. A pathologic complete response to preoperative chemoradiation is associated with lower local recurrence and improved survival in rectal cancer patients treated by mesorectal excision. *Dis Colon Rectum* 2003; 46: 298-304

García-Solano J, Pérez-Guillermo M, Conesa-Zamora P, Acosta-Ortega J, Trujillo-Santos J, Cerezuela-Fuentes P and Mäkinen MJ. Clinicopathologic study of 85 colorectal serrated adenocarcinomas: further insights into the full recognition of a new subset of colorectal carcinoma. *Hum Pathol* 2010; 41: 1359-68

Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U and Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol* 1984; 133: 1710-5

Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, Martinez P, Matthews N, Stewart A, Tarpey P, Varela I, Phillimore B, Begum S, McDonald NQ, Butler A, Jones D, Raine K, Latimer C, Santos CR, Nohadani M, Eklund AC, Spencer-Dene B, Clark G, Pickering L, Stamp G, Gore M, Szallasi Z, Downward J, Futreal PA and Swanton C. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med* 2012; 366: 883-92

Giardiello FM, Hamilton SR, Krush AJ, Piantadosi S, Hylind LM, Celano P, Booker SV, Robinson CR and Offerhaus GJ. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. *N Engl J Med* 1993; 328: 1313-6

Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, Jacquemier J, Viens P, Kleer CG, Liu S, Schott A, Hayes D, Birnbaum D, Wicha MS and Dontu G. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell* 2007; 1: 555-67

Giralt J, de las Heras M, Cerezo L, Eraso A, Hermosilla E, Velez D, Lujan J, Espin E, Rosello J, Majó J, Benavente S, Armengol M, de Torres I; Grupo Español de Investigacion Clinica en Oncologia Radioterápica (GICOR). The expression of epidermal growth factor receptor results in a worse prognosis for patients with rectal cancer treated with preoperative radiotherapy: a multicenter, retrospective analysis. *Radiother Oncol* 2005; 74: 101-8

Glimelius B and Cavalli-Björkman N. Metastatic colorectal cancer: current treatment and future options for improved survival. Medical approach--present status. *Scand J Gastroenterol* 2012; 47: 296-314

Gorham H, Sugino T, Woodman AC and Tarin D. Cellular distribution of CD44 gene transcripts in colorectal carcinomas and in normal colonic mucosa. *J Clin Pathol* 1996; 49: 482-8

Gosens MJ, van Kempen LC, van de Velde CJ, van Krieken JH and Nagtegaal ID. Loss of membranous Ep-CAM in budding colorectal carcinoma cells. *Mod Pathol* 2007; 20: 221-32

Gray R, Barnwell J, McConkey C, Hills RK, Williams NS, Kerr DJ and Group QC. Adjuvant chemotherapy versus observation in patients with colorectal cancer: a randomised study. *Lancet* 2007; 370: 2020-9

Greenhough A, Smartt HJ, Moore AE, Roberts HR, Williams AC, Paraskeva C and Kaidi A. The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. *Carcinogenesis* 2009; 30: 377-86

Greystoke A and Mullamitha SA. How many diseases are colorectal cancer? *Gastroenterol Res Pract* 2012; 2012: 564741

Group COoSTS. A comparison of laparoscopically assisted and open colectomy for colon cancer. *N Engl J Med* 2004; 350: 2050-9

Grönroos JO, Laine VJ and Nevalainen TJ. Bactericidal group IIA phospholipase A2 in serum of patients with bacterial infections. *J Infect Dis* 2002; 185: 1767-72

Gschwind A, Fischer OM and Ullrich A. The discovery of receptor tyrosine kinases: targets for cancer therapy. *Nat Rev Cancer* 2004; 4: 361-70

Gunderson LL, Jessup JM, Sargent DJ, Greene FL and Stewart A. Revised tumor and node categorization for rectal cancer based on surveillance, epidemiology, and end results and rectal pooled analysis outcomes. *J Clin Oncol* 2010a; 28: 256-63

Gunderson LL, Jessup JM, Sargent DJ, Greene FL and Stewart AK. Revised TN categorization for colon cancer based on national survival outcomes data. *J Clin Oncol* 2010b; 28: 264-71

Günthert U, Hofmann M, Rudy W, Reber S, Zöller M, Haussmann I, Matzku S, Wenzel A, Ponta H and Herrlich P. A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. *Cell* 1991; 65: 13-24

Haapamäki MM, Grönroos JM, Nurmi H, Alanen K, Kallajoki M and Nevalainen TJ. Gene expression of group II phospholipase A2 in intestine in ulcerative colitis. *Gut* 1997; 40: 95-101

Habr-Gama A, Perez RO, Nadalin W, Sabbaga J, Ribeiro U, Silva e Sousa AH, Campos FG, Kiss DR and Gama-Rodrigues J. Operative versus nonoperative treatment for stage 0 distal rectal cancer following chemoradiation therapy: long-term results. *Ann Surg* 2004; 240: 711-7; discussion 717-8

Halloran S, Launoy G, Zappa M: Chapter 4: Faecal Occult Blood Testing *in* Segnan N, Patnick J, von Karsa L (eds): European guidelines for quality assurance in colorectal cancer screening and diagnosis-First edition. European Comission, Publications Office of the European Union, Luxembourg, 2010

Hamilton SR, Bosman FT, Boffetta P, Ilyas M, Morreau H, Nakamura SI, Quirke P, Riboli E, Sobin LH: Carcinoma of the colon and rectum *in* Bosman FT, Carneiro F, Hruban RH, Theise ND (eds): WHO Classification of Tumours of the Digestive System, 4th edition, International Agency for Research on Cancer, Lyon, 2010, pp 134-46

Hamilton W and Sharp D. Diagnosis of colorectal cancer in primary care: the evidence base for guidelines. *Fam Pract* 2004; 21: 99-106

Hanahan D and Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; 144: 646-74

Hansen TF, Christensen R, Andersen RF, Garm Spindler KL, Johnsson A and Jakobsen A. The predictive value of single nucleotide polymorphisms in the VEGF system to the efficacy of first-line treatment with bevacizumab plus chemotherapy in patients with metastatic colorectal cancer: results from the Nordic ACT trial. *Int J Colorectal Dis* 2012; 27: 715-20

Hawkins NJ and Ward RL. Sporadic colorectal cancers with microsatellite instability and their possible origin in hyperplastic polyps and serrated adenomas. *J Natl Cancer Inst* 2001; 93: 1307-13

Haydon AM and Jass JR. Emerging pathways in colorectal-cancer development. *Lancet Oncol* 2002; 3: 83-8

Heald RJ and Ryall RD. Recurrence and survival after total mesorectal excision for rectal cancer. *Lancet* 1986; 1: 1479-82

Heaney AP, Nelson V, Fernando M and Horwitz G. Transforming events in thyroid tumorigenesis and their association with follicular lesions. *J Clin Endocrinol Metab* 2001; 86: 5025-32

Heaney AP, Singson R, McCabe CJ, Nelson V, Nakashima M and Melmed S. Expression of pituitary-tumour transforming gene in colorectal tumours. *Lancet* 2000; 355: 716-9

Hernández M, Martín R, García-Cubillas MD, Maeso-Hernández P and Nieto ML. Secreted PLA2 induces proliferation in astrocytoma through the EGF receptor: another inflammation-cancer link. *Neuro Oncol* 2010; 12: 1014-23

Herrlich P, Morrison H, Sleeman J, Orian-Rousseau V, König H, Weg-Remers S and Ponta H. CD44 acts both as a growth- and invasiveness-promoting molecule and as a tumor-suppressing cofactor. *Ann N Y Acad Sci* 2000; 910: 106-18; discussion 118-20

Hessman CJ, Bubbers EJ, Billingsley KG, Herzig DO and Wong MH. Loss of expression of the cancer stem cell marker aldehyde dehydrogenase 1 correlates with advanced-stage colorectal cancer. *Am J Surg* 2012; 203: 649-53

Hilska M, Collan YU, O Laine VJ, Kössi J, Hirsimäki P, Laato M and Roberts PJ. The significance of tumor markers for proliferation and apoptosis in predicting survival in colorectal cancer. *Dis Colon Rectum* 2005; 48: 2197-208

Hlubek F, Pfeiffer S, Budczies J, Spaderna S, Jung A, Kirchner T and Brabletz T. Securin (hPTTG1) expression is regulated by beta-catenin/TCF in human colorectal carcinoma. *Br J Cancer* 2006; 94: 1672-7

Hodgson DC, Fuchs CS and Ayanian JZ. Impact of patient and provider characteristics on the treatment and outcomes of colorectal cancer. *J Natl Cancer Inst* 2001; 93: 501-15

Hofheinz RD, Wenz F, Post S, Matzdorff A, Laechelt S, Hartmann JT, Müller L, Link H, Moehler M, Kettner E, Fritz E, Hieber U, Lindemann HW, Grunewald M, Kremers S, Constantin C, Hipp M, Hartung G, Gencer D, Kienle P, Burkholder I and Hochhaus A. Chemoradiotherapy with capecitabine versus fluorouracil for locally advanced rectal cancer: a randomised, multicentre, non-inferiority, phase 3 trial. *Lancet Oncol* 2012; 13: 579-88

Holm T, Ljung A, Häggmark T, Jurell G and Lagergren J. Extended abdominoperineal resection with gluteus maximus flap reconstruction of the pelvic floor for rectal cancer. *Br J Surg* 2007; 94: 232-8

Horst D, Kriegl L, Engel J, Kirchner T and Jung A. Prognostic significance of the cancer stem cell markers CD133, CD44, and CD166 in colorectal cancer. *Cancer Invest* 2009; 27: 844-50

Howlader N, Noone AM, Krapcho M, Neyman N, Aminou R, Waldron W, Altekruse SF, Kosary CL, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Eisner MP, Lewis DR, Chen HS, Feuer EJ, Cronin A (eds). SEER Cancer Statistics Review, 1975-2009 (Vintage 2009 Publications)**,** National Cancer Institute, Bethesda, MD, http://seer.cancer.gov/csr/1975_2009_pops09/, based on November 2011 SEER data submission, posted to the SEER web site, April 2012

Hsu LC, Chang WC and Yoshida A. Genomic structure of the human cytosolic aldehyde dehydrogenase gene. *Genomics* 1989; 5: 857-65

Huang EH, Hynes MJ, Zhang T, Ginestier C, Dontu G, Appelman H, Fields JZ, Wicha MS and Boman BM. Aldehyde dehydrogenase 1 is a marker for normal and malignant human colonic stem cells (SC) and tracks SC overpopulation during colon tumorigenesis. Cancer Res 2009; 69: 3382-9

Huerta S, Gao X and Saha D. Mechanisms of resistance to ionizing radiation in rectal cancer. *Expert Rev Mol Diagn* 2009; 9: 469-80

Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R and Kabbinavar F. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* 2004; 350: 2335-42

Huxley RR, Ansary-Moghaddam A, Clifton P, Czernichow S, Parr CL and Woodward M. The impact of dietary and lifestyle risk factors on risk of colorectal cancer: a quantitative overview of the epidemiological evidence. *Int J Cancer* 2009; 125: 171-80

Hörkkö TT, Klintrup K, Mäkinen JM, Näpänkangas JB, Tuominen HJ, Mäkelä J, Karttunen TJ and Mäkinen MJ. Budding invasive margin and prognosis in colorectal cancer--no direct association with beta-catenin expression. *Eur J Cancer* 2006; 42: 964-71

Ichii S, Horii A, Nakatsuru S, Furuyama J, Utsunomiya J and Nakamura Y. Inactivation of both APC alleles in an early stage of colon adenomas in a patient with familial adenomatous polyposis (FAP). *Hum Mol Genet* 1992; 1: 387-90

Improved survival with preoperative radiotherapy in resectable rectal cancer. Swedish Rectal Cancer Trial. *N Engl J Med* 1997; 336: 980-7

Inno A, Di Salvatore M, Cenci T, Martini M, Orlandi A, Strippoli A, Ferrara AM, Bagalà C, Cassano A, Larocca LM and Barone C. Is there a role for IGF1R and c-MET pathways in resistance to cetuximab in metastatic colorectal cancer? *Clin Colorectal Cancer* 2011; 10: 325-32

International Union Against Cancer (UICC). Sobin L, Wittekind C (eds). Colon and Rectum *in* TNM classification of malignant tumours, 6th edition, 2002, Wiley-Liss, New York, NY, pp 72-6

International Union Against Cancer (UICC). Sobin L, Gospodarowicz M, Wittekind C (eds). Colon and Rectum i*n* TNM classification of malignant tumours,7th edition, 2010, Wiley-Blackwell, Singapore, pp 100-5

Ishida T. Immunohistochemical expression of the CD44 variant 6 in colorectal adenocarcinoma. *Surg Today* 2000; 30: 28-32

Jakob C, Liersch T, Meyer W, Becker H, Baretton GB and Aust DE. Predictive value of Ki67 and p53 in locally advanced rectal cancer: correlation with thymidylate synthase and histopathological tumor regression after neoadjuvant 5-FU-based chemoradiotherapy. *World J Gastroenterol* 2008; 14: 1060-6

Jallepalli PV, Waizenegger IC, Bunz F, Langer S, Speicher MR, Peters JM, Kinzler KW, Vogelstein B and Lengauer C. Securin is required for chromosomal stability in human cells. *Cell* 2001; 105: 445-57

Janjan NA, Khoo VS, Abbruzzese J, Pazdur R, Dubrow R, Cleary KR, Allen PK, Lynch PM, Glober G, Wolff R, Rich TA and Skibber J. Tumor downstaging and sphincter preservation with preoperative chemoradiation in locally advanced rectal cancer: the M. D. Anderson Cancer Center experience. *Int J Radiat Oncol Biol Phys* 1999; 44: 1027-38

Janson ET, Sørbye H, Welin S, Federspiel B, Grønbaek H, Hellman P, Mathisen O, Mortensen J, Sundin A, Thiis-Evensen E, Välimäki MJ, Oberg K and Knigge U. Nordic Guidelines 2010 for diagnosis and treatment of gastroenteropancreatic neuroendocrine tumours. *Acta Oncol* 2010; 49: 740-56

Jass JR, Atkin WS, Cuzick J, Bussey HJ, Morson BC, Northover JM and Todd IP. The grading of rectal cancer: historical perspectives and a multivariate analysis of 447 cases. *Histopathology* 1986; 10: 437-59

Jass JR, Love SB and Northover JM. A new prognostic classification of rectal cancer. *Lancet* 1987; 1: 1303-6

Jayne DG, Thorpe HC, Copeland J, Quirke P, Brown JM and Guillou PJ. Five-year follow-up of the Medical Research Council CLASICC trial of laparoscopically assisted versus open surgery for colorectal cancer. *Br J Surg* 2010; 97: 1638-45

Jensen NF, Smith DH, Nygård SB, Rømer MU, Nielsen KV and Brünner N. Predictive biomarkers with potential of converting conventional chemotherapy to targeted therapy in patients with metastatic colorectal cancer. *Scand J Gastroenterol* 2012; 47: 340-55

Jernvall P, Mäkinen MJ, Karttunen TJ, Mäkelä J and Vihko P. Microsatellite instability: impact on cancer progression in proximal and distal colorectal cancers. *Eur J Cancer* 1999; 35: 197-201

Ji BT, Weissfeld JL, Chow WH, Huang WY, Schoen RE, Hayes RB. Tobacco smoking and colorectal hyperplastic and adenomatous polyps. *Cancer Epidemiol Biomarkers Prev* 2006; 15: 897-901

Jiang F, Qiu Q, Khanna A, Todd NW, Deepak J, Xing L, Wang H, Liu Z, Su Y, Stass SA and Katz RL. Aldehyde dehydrogenase 1 is a tumor stem cell-associated marker in lung cancer. *Mol Cancer Res* 2009; 7: 330-8

Järvinen HJ, Renkonen-Sinisalo L, Aktán-Collán K, Peltomäki P, Aaltonen LA and Mecklin JP. Ten years after mutation testing for Lynch syndrome: cancer incidence and outcome in mutation-positive and mutation-negative family members. *J Clin Oncol* 2009; 27: 4793-7

Kahlert C, Bergmann F, Beck J, Welsch T, Mogler C, Herpel E, Dutta S, Niemietz T, Koch M and Weitz

J. Low expression of aldehyde dehydrogenase 1A1 (ALDH1A1) is a prognostic marker for poor survival in pancreatic cancer. *BMC Cancer* 2011; 11: 275

Kahlert C, Gaitzsch E, Steinert G, Mogler C, Herpel E, Hoffmeister M, Jansen L, Benner A, Brenner H, Chang-Claude J, Rahbari N, Schmidt T, Klupp F, Grabe N, Lahrmann B, Koch M, Halama N, Büchler M and Weitz J. Expression Analysis of Aldehyde Dehydrogenase 1A1 (ALDH1A1) in Colon and Rectal Cancer in Association with Prognosis and Response to Chemotherapy. *Ann Surg Oncol* 2012

Kakar SS and Jennes L. Molecular cloning and characterization of the tumor transforming gene (TUTR1): a novel gene in human tumorigenesis. *Cytogenet Cell Genet* 1999; 84: 211-6

Kalady MF, de Campos-Lobato LF, Stocchi L, Geisler DP, Dietz D, Lavery IC and Fazio VW. Predictive factors of pathologic complete response after neoadjuvant chemoradiation for rectal cancer. *Ann Surg* 2009; 250: 582-9

Kapiteijn E, Marijnen CA, Nagtegaal ID, Putter H, Steup WH, Wiggers T, Rutten HJ, Pahlman L, Glimelius B, van Krieken JH, Leer JW, van de Velde CJ and Group DCC. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer. *N Engl J Med* 2001; 345: 638-46

Karameris A, Kanavaros P, Aninos D, Gorgoulis V, Mikou G, Rokas T, Niotis M and Kalogeropoulos N. Expression of epidermal growth factor (EGF) and epidermal growth factor receptor (EGFR) in gastric and colorectal carcinomas. An immunohistological study of 63 cases. *Pathol Res Pract* 1993;189:133-7

Kasper S, Breitenbuecher F, Reis H, Brandau S, Worm K, Köhler J, Paul A, Trarbach T, Schmid KW and Schuler M. Oncogenic RAS simultaneously protects against anti-EGFR antibody-dependent cellular cytotoxicity and EGFR signaling blockade. *Oncogene* 2012

Kennedy BP, Soravia C, Moffat J, Xia L, Hiruki T, Collins S, Gallinger S and Bapat B. Overexpression of the nonpancreatic secretory group II PLA2 messenger RNA and protein in colorectal adenomas from familial adenomatous polyposis patients. *Cancer Res* 1998; 58: 500-3

Kim DS, Franklyn JA, Smith VE, Stratford AL, Pemberton HN, Warfield A, Watkinson JC, Ishmail T, Wakelam MJ and McCabe CJ. Securin induces genetic instability in colorectal cancer by inhibiting double-stranded DNA repair activity. *Carcinogenesis* 2007; 28: 749-59

Kim MA, Lee HJ, Yang HK, Bang YJ and Kim WH. Heterogeneous amplification of ERBB2 in primary lesions is responsible for the discordant ERBB2 status of primary and metastatic lesions in gastric carcinoma. *Histopathology* 2011; 59: 822-31

Kinzler KW and Vogelstein B. Lessons from hereditary colorectal cancer. *Cell* 1996; 87: 159-70

Kobayashi H, Mochizuki H, Kato T, Mori T, Kameoka S, Shirouzu K, Saito Y, Watanabe M, Morita T, Hida J, Ueno M, Ono M, Yasuno M and Sugihara K. Is total mesorectal excision always necessary for T1-T2 lower rectal cancer? *Ann Surg Oncol* 2010; 17: 973-80

Kopetz S, Chang GJ, Overman MJ, Eng C, Sargent DJ, Larson DW, Grothey A, Vauthey JN, Nagorney DM and McWilliams RR. Improved survival in metastatic colorectal cancer is associated with adoption of hepatic resection and improved chemotherapy. *J Clin Oncol* 2009; 27: 3677-83

Korinek V, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW, Vogelstein B and Clevers H. Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. *Science* 1997; 275: 1784-7

Korkeila E, Jaakkola PM, Syrjänen K, Pyrhönen S and Sundström J. Pronounced tumour regression after radiotherapy is associated with negative/weak glucose transporter-1 expression in rectal cancer. *Anticancer Res* 2011; 31: 311-5

Korkeila E, Sundström J, Pyrhönen S and Syrjänen K. Main effects and interactions of carbonic anhydrase IX, hypoxia-inducible factor-1α, ezrin and glucose transporter-1 in multivariate analysis for disease outcome in rectal cancer. *Anticancer Res* 2012; 32: 3299-303

Korkeila E, Talvinen K, Jaakkola PM, Minn H, Syrjänen K, Sundström J and Pyrhönen S. Expression of carbonic anhydrase IX suggests poor outcome in rectal cancer. *Br J Cancer* 2009; 100: 874-80

Koukourakis MI, Giatromanolaki A, Tsakmaki V, Danielidis V and Sivridis E. Cancer stem cell phenotype relates to radio-chemotherapy outcome in locally advanced squamous cell head-neck cancer. *Br J Cancer* 2012; 106: 846-53

Kouri M. DNA ploidy of colorectal carcinoma by tumour site, gender and history of noncolorectal malignancies. *Oncology* 1993; 50: 41-5

Kouri M, Pyrhönen S and Kuusela P. Elevated CA19-9 as the most significant prognostic factor in advanced colorectal carcinoma. *J Surg Oncol* 1992; 49: 78-85

Kramer RM, Hession C, Johansen B, Hayes G, McGray P, Chow EP, Tizard R and Pepinsky RB. Structure and properties of a human non-pancreatic phospholipase A2. *J Biol Chem* 1989; 264: 5768-75

Kraus C, Liehr T, Hülsken J, Behrens J, Birchmeier W, Grzeschik KH and Ballhausen WG. Localization of the human beta-catenin gene (CTNNB1) to 3p21: a region implicated in tumor development. *Genomics* 1994; 23: 272-4

Kressner U, Lindmark G, Gerdin B, Pahlman L and Glimelius B. Heterogeneity in proliferation markers in colorectal cancer. *Anticancer Res* 1995; 15: 2755-61

Kuremsky JG, Tepper JE and McLeod HL. Biomarkers for response to neoadjuvant chemoradiation for rectal cancer. *Int J Radiat Oncol Biol Phys* 2009; 74: 673-88

Lacy AM, García-Valdecasas JC, Delgado S, Castells A, Taurá P, Piqué JM and Visa J. Laparoscopy-assisted colectomy versus open colectomy for treatment of non-metastatic colon cancer: a randomised trial. *Lancet* 2002; 359: 2224-9

Lahaye MJ, Engelen SM, Nelemans PJ, Beets GL, van de Velde CJ, van Engelshoven JM and Beets-Tan RG. Imaging for predicting the risk factors--the circumferential resection margin and nodal disease--of local recurrence in rectal cancer: a meta-analysis. *Semin Ultrasound CT MR* 2005; 26: 259-68

Lahaye MJ, Engelen SM, Kessels AG, de Bruïne AP, von Meyenfeldt MF, van Engelshoven JM, van de Velde CJ, Beets GL, Beets-Tan RG. USPIO-enhanced MR imaging for nodal staging in patients with primary rectal cancer: predictive criteria. *Radiology* 2008; 246: 804-11

Laine VJ, Grass DS and Nevalainen TJ. Protection by group II phospholipase A2 against Staphylococcus aureus. *J Immunol* 1999; 162: 7402-8

Lambrecht M, Deroose C, Roels S, Vandecaveye V, Penninckx F, Sagaert X, van Cutsem E, de Keyzer F and Haustermans K. The use of FDG-PET/CT and diffusion-weighted magnetic resonance imaging for response prediction before, during and after preoperative chemoradiotherapy for rectal cancer. *Acta Oncol* 2010; 49: 956-63

Lange MM, Rutten HJ and van de Velde CJ. One hundred years of curative surgery for rectal cancer: 1908-2008. *Eur J Surg Oncol* 2009; 35: 456-63

Le NH, Franken P and Fodde R. Tumour-stroma interactions in colorectal cancer: converging on beta-catenin activation and cancer stemness. *Br J Cancer* 2008; 98: 1886-93

Leidy C, Linderoth L, Andresen TL, Mouritsen OG, Jørgensen K and Peters GH. Domain-induced activation of human phospholipase A2 type IIA: local versus global lipid composition. *Biophys J* 2006; 90: 3165-75

Lepistö A, Österlund P, Kouri M and Järvinen HJ. Peräsuolen syöpä. *Lääketieteellinen Aikakauskirja Duodecim* 2009;125:857- 65

Lesley J, Hyman R and Kincade PW. CD44 and its interaction with extracellular matrix. *Adv Immuno*l 1993; 54: 271-335

Li FY and Lai MD. Colorectal cancer, one entity or three. *J Zhejiang Univ Sci B* 2009; 10: 219-29

Liang D and Shi Y. Aldehyde dehydrogenase-1 is a specific marker for stem cells in human lung adenocarcinoma. *Med Oncol* 2012; 29: 633-9

Liang PS, Chen TY and Giovannucci E. Cigarette smoking and colorectal cancer incidence and mortality: systematic review and meta-analysis. *Int J Cancer* 2009; 124: 2406-15

Liebig C, Ayala G, Wilks J, Verstovsek G, Liu H, Agarwal N, Berger DH and Albo D. Perineural invasion is an independent predictor of outcome in colorectal cancer. *J Clin Oncol* 2009; 27: 5131-7

Lièvre A, Bachet JB, Le Corre D, Boige V, Landi B, Emile JF, Côté JF, Tomasic G, Penna C, Ducreux M, Rougier P, Penault-Llorca F and Laurent-Puig P. KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. *Cancer Res* 2006; 66: 3992-5

Lohberger B, Rinner B, Stuendl N, Absenger M, Liegl-Atzwanger B, Walzer SM, Windhager R and Leithner A. Aldehyde dehydrogenase 1, a potential marker for cancer stem cells in human sarcoma. *PLoS One* 2012; 7: e43664

Long MD, Martin CF, Galanko JA and Sandler RS. Hormone replacement therapy, oral contraceptive use, and distal large bowel cancer: a population-based case-control study. *Am J Gastroenterol* 2010; 105: 1843-50

Lugli A, Iezzi G, Hostettler I, Muraro MG, Mele V, Tornillo L, Carafa V, Spagnoli G, Terracciano L and Zlobec I. Prognostic impact of the expression of putative cancer stem cell markers CD133, CD166, CD44s, EpCAM, and ALDH1 in colorectal cancer. *Br J Cancer* 2010; 103: 382-90

Lynch HT, Lynch JF, Lynch PM and Attard T. Hereditary colorectal cancer syndromes: molecular genetics, genetic counseling, diagnosis and management. *Fam Cancer* 2008; 7: 27-39

MacPhee M, Chepenik KP, Liddell RA, Nelson KK, Siracusa LD and Buchberg AM. The secretory phospholipase A2 gene is a candidate for the Mom1 locus, a major modifier of ApcMin-induced intestinal neoplasia. *Cell* 1995; 81: 957-66

Malik MT and Kakar SS. Regulation of angiogenesis and invasion by human Pituitary tumor transforming gene (PTTG) through increased expression and secretion of matrix metalloproteinase-2 (MMP-2). *Mol Cancer* 2006; 5: 61

Malila N, Palva T, Malminiemi O, Paimela H, Anttila A, Hakulinen T, Järvinen H, Kotisaari ML, Pikkarainen P, Rautalahti M, Sankila R, Vertio H and Hakama M. Coverage and performance of colorectal cancer screening with the faecal occult blood test in Finland. *J Med Screen* 2011; 18: 18-23

Marcato P, Dean CA, Giacomantonio CA and Lee PW. Aldehyde dehydrogenase: its role as a cancer stem

cell marker comes down to the specific isoform. *Cell Cycle* 2011; 10: 1378-84

Marchitti SA, Brocker C, Stagos D and Vasiliou V. Non-P450 aldehyde oxidizing enzymes: the aldehyde dehydrogenase superfamily. E*xpert Opin Drug Metab Toxicol* 2008; 4: 697-720

Marijnen CA, Nagtegaal ID, Klein Kranenbarg E, Hermans J, van de Velde CJ, Leer JW, van Krieken JH and Investigators PRCatCC. No downstaging after short-term preoperative radiotherapy in rectal cancer patients. *J Clin Oncol* 2001; 19: 1976-84

Markowitz SD and Bertagnolli MM. Molecular origins of cancer: Molecular basis of colorectal cancer. *N Engl J Med* 2009; 361: 2449-60

Marr R, Birbeck K, Garvican J, Macklin CP, Tiffin NJ, Parsons WJ, Dixon MF, Mapstone NP, Sebag-Montefiore D, Scott N, Johnston D, Sagar P, Finan P and Quirke P. The modern abdominoperineal excision: the next challenge after total mesorectal excision. *Ann Surg* 2005; 242: 74-82

Marusyk A, Almendro V and Polyak K. Intra-tumour heterogeneity: a looking glass for cancer? *Nat Rev Cancer* 2012; 12: 323-34

McCabe CJ, Boelaert K, Tannahill LA, Heaney AP, Stratford AL, Khaira JS, Hussain S, Sheppard MC, Franklyn JA and Gittoes NJ. Vascular endothelial growth factor, its receptor KDR/Flk-1, and pituitary tumor transforming gene in pituitary tumors. *J Clin Endocrinol Metab* 2002; 87: 4238-44

McCabe CJ and Heaney AP. Pituitary tumour transforming gene in endocrine cancer. *Clin Endocrinol (Oxf)* 2003; 58: 673-82

Mecklin JP, Aarnio M, Läärä E, Kairaluoma MV, Pylvänäinen K, Peltomäki P, Aaltonen LA and Järvinen HJ. Development of colorectal tumors in colonoscopic surveillance in Lynch syndrome. *Gastroenterology* 2007; 133: 1093-8

MERCURY Study Group. Extramural depth of tumor invasion at thin-section MR in patients with rectal cancer: results of the MERCURY study. *Radiology* 2007; 243: 132-9

Metzger B, Chambeau L, Begon DY, Faber C, Kayser J, Berchem G, Pauly M, Boniver J, Delvenne P, Dicato M and Wenner T. The human epidermal growth factor receptor (EGFR) gene in European patients with advanced colorectal cancer harbors infrequent mutations in its tyrosine kinase domain. *BMC Med Genet* 2011; 12: 144

Meyerhardt JA and Mayer RJ. Systemic therapy for colorectal cancer. *N Engl J Med* 2005; 352: 476-87

Mihatsch J, Toulany M, Bareiss PM, Grimm S, Lengerke C, Kehlbach R and Rodemann HP. Selection of radioresistant tumor cells and presence of ALDH1 activity in vitro. *Radiother Oncol* 2011; 99: 300-6

Minoo P, Zlobec I, Peterson M, Terracciano L and Lugli A. Characterization of rectal, proximal and distal colon cancers based on clinicopathological, molecular and protein profiles. *Int J Oncol* 2010; 37: 707-18

Misale S, Yaeger R, Hobor S, Scala E, Janakiraman M, Liska D, Valtorta E, Schiavo R, Buscarino M, Siravegna G, Bencardino K, Cercek A, Chen CT, Veronese S, Zanon C, Sartore-Bianchi A, Gambacorta M, Gallicchio M, Vakiani E, Boscaro V, Medico E, Weiser M, Siena S, Di Nicolantonio F, Solit D and Bardelli A. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. *Nature* 2012; 486: 532-6

Misra S, Heldin P, Hascall VC, Karamanos NK, Skandalis SS, Markwald RR and Ghatak S. Hyaluronan-CD44 interactions as potential targets for cancer therapy. *FEBS J* 2011; 278: 1429-43

Miyoshi Y, Nagase H, Ando H, Horii A, Ichii S, Nakatsuru S, Aoki T, Miki Y, Mori T and Nakamura Y. Somatic mutations of the APC gene in colorectal tumors: mutation cluster region in the APC gene. *Hum Mol Genet* 1992; 1: 229-33

Moertel CG, Fleming TR, Macdonald JS, Haller DG, Laurie JA, Goodman PJ, Ungerleider JS, Emerson WA, Tormey DC and Glick JH. Levamisole and fluorouracil for adjuvant therapy of resected colon carcinoma. *N Engl J Med* 1990; 322: 352-8

Mora-Santos M, Castilla C, Herrero-Ruiz J, Giráldez S, Limón-Mortés MC, Sáez C, Japón MA, Tortolero M and Romero F. A single mutation in Securin induces chromosomal instability and enhances cell invasion. *Eur J Cancer* 2012

Moroni M, Veronese S, Benvenuti S, Marrapese G, Sartore-Bianchi A, Di Nicolantonio F, Gambacorta M, Siena S and Bardelli A. Gene copy number for epidermal growth factor receptor (EGFR) and clinical response to antiEGFR treatment in colorectal cancer: a cohort study. *Lancet Oncol* 2005; 6: 279-86

Morrin M and Delaney PV. CD44v6 is not relevant in colorectal tumour progression. *Int J Colorectal Dis* 2002; 17: 30-6

Moureau-Zabotto L, Farnault B, de Chaisemartin C, Esterni B, Lelong B, Viret F, Giovannini M, Monges G, Delpero JR, Bories E, Turrini O, Viens P and Salem N. Predictive factors of tumor response after neoadjuvant chemoradiation for locally advanced rectal cancer. *Int J Radiat Oncol Biol Phys* 2011; 80: 483-91

Mulder JW, Kruyt PM, Sewnath M, Oosting J, Seldenrijk CA, Weidema WF, Offerhaus GJ and Pals ST. Colorectal cancer prognosis and expression of exon-v6-containing CD44 proteins. *Lancet* 1994; 344: 1470-2

Mysliwiec PA, Cronin KA, Schatzkin A: Chapter 5: New malignancies following cancer of the colon, rectum, and anus *in* Curtis RE, Freedman DM, Ron E, Ries LAG, Hacker DG, Edwards BK, Tucker MA, Fraumeni JF Jr (eds): New malignancies among cancer survivors: SEER Cancer Registries, 1973-2000. National Cancer Institute, NIH Publ. No. 05-5302, Bethesda, MD, 2006

Mäkinen MJ. Colorectal serrated adenocarcinoma. *Histopathology* 2007; 50: 131-50

Nagtegaal ID, Marijnen CA, Kranenbarg EK, Mulder-Stapel A, Hermans J, van de Velde CJ, van Krieken JH and Committee PR. Short-term preoperative radiotherapy interferes with the determination of pathological parameters in rectal cancer. *J Pathol* 2002; 197: 20-7

Nagtegaal ID and Quirke P. What is the role for the circumferential margin in the modern treatment of rectal cancer? J Clin Oncol 2008; 26: 303-12

National Cancer Institut. NCI Dictionary on Cancer Terms. www.cancer.gov/dictionary

Naor D, Nedvetzki S, Golan I, Melnik L and Faitelson Y. CD44 in cancer. *Crit Rev Clin Lab Sci* 2002; 39: 527-79

Nevalainen TJ, Eerola LI, Rintala E, Laine VJ, Lambeau G and Gelb MH. Time-resolved fluoroimmunoassays of the complete set of secreted phospholipases A2 in human serum. *Biochim Biophys Acta* 2005; 1733: 210-23

Nevalainen TJ, Grönroos JM and Kallajoki M. Expression of group II phospholipase A2 in the human gastrointestinal tract. *Lab Invest* 1995 ;72: 201-8

Nevalainen TJ and Haapanen TJ. Distribution of pancreatic (group I) and synovial-type (group II) phospholipases A2 in human tissues. *Inflammation* 1993; 17: 453-64

Nevalainen TJ, Märki F, Kortesuo PT, Grütter MG, Di Marco S and Schmitz A. Synovial type (group II) phospholipase A2 in cartilage. *J Rheumatol* 1993; 20: 325-30

Niikura N, Liu J, Hayashi N, Mittendorf EA, Gong Y, Palla SL, Tokuda Y, Gonzalez-Angulo AM, Hortobagyi GN and Ueno NT. Loss of human epidermal growth factor receptor 2 (HER2) expression in metastatic sites of HER2-overexpressing primary breast tumors. *J Clin Onco*l 2012; 30: 593-9

Nimmrich I, Friedl W, Kruse R, Pietsch S, Hentsch S, Deuter R, Winde G and Müller O. Loss of the PLA2G2A gene in a sporadic colorectal tumor of a patient with a PLA2G2A germline mutation and absence of PLA2G2A germline alterations in patients with FAP. *Hum Genet* 1997; 100: 345-9

Nyasavajjala SM, Shaw AG, Khan AQ, Brown SR and Lund JN. Neoadjuvant chemo-radiotherapy and rectal cancer: can the UK watch and wait with Brazil? *Colorectal Dis* 2010; 12: 33-6

Oh PS, Patel VB, Sanders MA, Kanwar SS, Yu Y, Nautiyal J, Patel BB and Majumdar AP. Schlafen-3 decreases cancer stem cell marker expression and autocrine/juxtacrine signaling in FOLFOX-resistant colon cancer cells. *Am J Physiol Gastrointest Liver Physiol* 2011; 301: G347-55

Orian-Rousseau V, Chen L, Sleeman JP, Herrlich P and Ponta H. CD44 is required for two consecutive steps in HGF/c-Met signaling. *Genes Dev* 2002; 16: 3074-86

Paimela H, Malila N, Palva T, Hakulinen T, Vertio H and Järvinen H. Early detection of colorectal cancer with faecal occult blood test screening. *Br J Surg* 2010; 97: 1567-71

Palmer G, Martling A, Cedermark B, Holm T. Preoperative tumour staging with multidisciplinary team assessment improves the outcome in locally advanced primary rectal cancer. *Colorectal Dis* 2011; 13: 1361-9

Parkin DM, Boyd L and Walker LC. 16. The fraction of cancer attributable to lifestyle and environmental factors in the UK in 2010. *Br J Cancer* 2011; 105 Suppl 2: S77-81

Pawlik TM and Keyomarsi K. Role of cell cycle in mediating sensitivity to radiotherapy. *Int J Radiat Oncol Biol Phys* 2004; 59: 928-42

Pei L and Melmed S. Isolation and characterization of a pituitary tumor-transforming gene (PTTG). *Mol Endocrinol* 1997; 11: 433-41

Peng J, Lu JJ, Zhu J, Xu Y, Lu H, Lian P, Cai G and Cai S. Prediction of treatment outcome by CD44v6 after total mesorectal excision in locally advanced rectal cancer. *Cancer J* 2008; 14: 54-61

Petersen SH, Harling H, Kirkeby LT, Wille-Jørgensen P, Mocellin S. Postoperative adjuvant treatment in rectal cancer operated for cure. *Cochrane Database Syst Rev* 2012; 3: CD004078

Pettersson D, Cedermark B, Holm T, Radu C, Påhlman L, Glimelius B and Martling A. Interim analysis of the Stockholm III trial of preoperative radiotherapy regimens for rectal cancer. *Br J Surg* 2010; 97: 580-7

Ponta H, Wainwright D and Herrlich P. The CD44 protein family. Int *J Biochem Cell Biol* 1998; 30: 299-305

Prolongation of the disease-free interval in surgically treated rectal carcinoma. Gastrointestinal Tumor Study Group. *N Engl J Med* 1985; 312: 1465-72

Quirke P, Durdey P, Dixon MF and Williams NS. Local recurrence of rectal adenocarcinoma due to inadequate surgical resection. Histopathological

study of lateral tumour spread and surgical excision. *Lancet* 1986; 2: 996-9

Quirke P, Risio M, Lambert R, von Karsa L and Vieth M. Quality assurance in pathology in colorectal cancer screening and diagnosis—European recommendations. *Virchows Arch* 2011; 458: 1-19

Radinsky R, Risin S, Fan D, Dong Z, Bielenberg D, Bucana CD and Fidler IJ. Level and function of epidermal growth factor receptor predict the metastatic potential of human colon carcinoma cells. *Clin Cancer Res* 1995; 1: 19-31

Renehan AG, Egger M, Saunders MP and O'Dwyer ST. Impact on survival of intensive follow up after curative resection for colorectal cancer: systematic review and meta-analysis of randomised trials. *BMJ* 2002; 324: 813

Renehan AG, Tyson M, Egger M, Heller RF and Zwahlen M. Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. *Lancet* 2008; 371: 569-78

Reya T, Morrison SJ, Clarke MF and Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* 2001; 414: 105-11

Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C and De Maria R. Identification and expansion of human colon-cancer-initiating cells. *Nature* 2007; 445: 111-5

Rich JN. Cancer stem cells in radiation resistance. *Cancer Res* 2007; 67: 8980-4

Riggins GJ, Markowitz S, Wilson JK, Vogelstein B and Kinzler KW. Absence of secretory phospholipase A2 gene alterations in human colorectal cancer. *Cancer Res* 1995; 55: 5184-6

Rintala EM and Nevalainen TJ. Group II phospholipase A2 in sera of febrile patients with microbiologically or clinically documented infections. *Clin Infect Dis* 1993; 17: 864-70

Risio M, Coverlizza S, Ferrari A, Candelaresi GL and Rossini FP. Immunohistochemical study of epithelial cell proliferation in hyperplastic polyps, adenomas, and adenocarcinomas of the large bowel. *Gastroenterology* 1988; 94: 899-906

Ropponen KM, Eskelinen MJ, Lipponen PK, Alhava E and Kosma VM. Expression of CD44 and variant proteins in human colorectal cancer and its relevance for prognosis. *Scand J Gastroenterol* 1998; 33: 301-9

Rothwell PM, Price JF, Fowkes FG, Zanchetti A, Roncaglioni MC, Tognoni G, Lee R, Belch JF, Wilson M, Mehta Z and Meade TW. Short-term effects of daily aspirin on cancer incidence, mortality, and non-vascular death: analysis of the time course of risks and benefits in 51 randomised controlled trials. *Lancet* 2012; 379: 1602-12

Rothwell PM, Wilson M, Price JF, Belch JF, Meade TW and Mehta Z. Effect of daily aspirin on risk of cancer metastasis: a study of incident cancers during randomised controlled trials. *Lancet* 2012; 379: 1591-601

Ryan R, Gibbons D, Hyland JM, Treanor D, White A, Mulcahy HE, O'Donoghue DP, Moriarty M, Fennelly D and Sheahan K. Pathological response following long-course neoadjuvant chemoradiotherapy for locally advanced rectal cancer. *Histopathology* 2005; 47: 141-6

Rödel C, Martus P, Papadoupolos T, Füzesi L, Klimpfinger M, Fietkau R, Liersch T, Hohenberger W, Raab R, Sauer R and Wittekind C. Prognostic significance of tumor regression after preoperative chemoradiotherapy for rectal cancer. *J Clin Oncol* 2005; 23: 8688-96

Salazar R, Roepman P, Capella G, Moreno V, Simon I, Dreezen C, Lopez-Doriga A, Santos C, Marijnen C, Westerga J, Bruin S, Kerr D, Kuppen P, van de Velde C, Morreau H, Van Velthuysen L, Glas AM, Van't Veer LJ and Tollenaar R. Gene expression signature to improve prognosis prediction of stage II and III colorectal cancer. *J Clin Oncol* 2011; 29: 17-24

Saltz LB, Cox JV, Blanke C, Rosen LS, Fehrenbacher L, Moore MJ, Maroun JA, Ackland SP, Locker PK, Pirotta N, Elfring GL and Miller LL. Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. Irinotecan Study Group. *N Engl J Med* 2000; 343: 905-14

Saltz LB, Niedzwiecki D, Hollis D, Goldberg RM, Hantel A, Thomas JP, Fields AL and Mayer RJ. Irinotecan fluorouracil plus leucovorin is not superior to fluorouracil plus leucovorin alone as adjuvant treatment for stage III colon cancer: results of CALGB 89803. *J Clin Oncol* 2007; 25: 3456-61

Sargent DJ, Marsoni S, Monges G, Thibodeau SN, Labianca R, Hamilton SR, French AJ, Kabat B, Foster NR, Torri V, Ribic C, Grothey A, Moore M, Zaniboni A, Seitz JF, Sinicrope F and Gallinger S. Defective mismatch repair as a predictive marker for lack of efficacy of fluorouracil-based adjuvant therapy in colon cancer. *J Clin Oncol* 2010; 28: 3219-26

Sauer R, Becker H, Hohenberger W, Rödel C, Wittekind C, Fietkau R, Martus P, Tschmelitsch J, Hager E, Hess CF, Karstens JH, Liersch T, Schmidberger H, Raab R and Group GRCS. Preoperative versus postoperative chemoradiotherapy for rectal cancer. *N Engl J Med* 2004; 351: 1731-40

Sauer R, Liersch T, Merkel S, Fietkau R, Hohenberger W, Hess C, Becker H, Raab HR, Villanueva MT, Witzigmann H, Wittekind C, Beissbarth T and Rödel C. Preoperative versus postoperative chemoradiotherapy for locally advanced rectal cancer: results of the German CAO/ARO/AIO-94

randomized phase III trial after a median follow-up of 11 years. *J Clin Oncol* 2012; 30: 1926-33

Saxena B, Sundaram ST, Walton W, Patel I, Kuo P, Khan S, Matathia A, Purohit A, Crowley R, Zhou Q; ImClone Systems, Branchburg, NJ. Differentiation between the EGFR antibodies necitumumab, cetuximab, and panitumumab: In vitro biological and binding activities. *J Clin Oncol* 2011**,** 29 suppl; ASCO meeting abstract abstr e13030

Scartozzi M, Bearzi I, Mandolesi A, Pierantoni C, Loupakis F, Zaniboni A, Negri F, Quadri A, Zorzi F, Galizia E, Berardi R, Biscotti T, Labianca R, Masi G, Falcone A and Cascinu S. Epidermal Growth Factor Receptor (EGFR) gene copy number (GCN) correlates with clinical activity of irinotecan-cetuximab in K-RAS wild-type colorectal cancer: a fluorescence in situ (FISH) and chromogenic in situ hybridization (CISH) analysis. *BMC Cancer* 2009; 9: 303

Schmoll HJ, Van Cutsem E, Stein A, Valentini V, Glimelius B, Haustermans K, Nordlinger B, van de Velde CJ, Balmana J, Regula J, Nagtegaal ID, Beets-Tan RG, Arnold D, Ciardiello F, Hoff P, Kerr D, Köhne CH, Labianca R, Price T, Scheithauer W, Sobrero A, Tabernero J, Aderka D, Barroso S, Bodoky G, Douillard JY, El Ghazaly H, Gallardo J, Garin A, Glynne-Jones R, Jordan K, Meshcheryakov A, Papamichail D, Pfeiffer P, Souglakos I, Turhal S and Cervantes A. ESMO Consensus Guidelines for management of patients with colon and rectal cancer. A personalized approach to clinical decision making. *Ann Oncol* 2012; 23: 2479-516

Sebag-Montefiore D, Stephens RJ, Steele R, Monson J, Grieve R, Khanna S, Quirke P, Couture J, de Metz C, Myint AS, Bessell E, Griffiths G, Thompson LC and Parmar M. Preoperative radiotherapy versus selective postoperative chemoradiotherapy in patients with rectal cancer (MRC CR07 and NCIC-CTG C016): a multicentre, randomised trial. *Lancet* 2009; 373: 811-20

Seufferlein T, Ahn J, Krndija D, Lother U, Adler G and von Wichert G. Tumor biology and cancer therapy - an evolving relationship. *Cell Commun Signal* 2009; 7: 19

Siegel R, Cuesta MA, Targarona E, Bader FG, Morino M, Corcelles R, Lacy AM, Påhlman L, Haglind E, Bujko K, Bruch HP, Heiss MM, Eikermann M, Neugebauer EA; European Association for Endoscopic Surgery (EAES). Laparoscopic extraperitoneal rectal cancer surgery: the clinical practice guidelines of the European Association for Endoscopic Surgery (EAES). *Surg Endosc* 2011; 25: 2423-40

Sigurdsson JA, Getz L, Sjönell G, Vainiomäki P and Brodersen J. Marginal public health gain of screening for colorectal cancer: modelling study, based on WHO and national databases in the Nordic countries. *J Eval Clin Pract* 2012

Sinicrope FA, Foster NR, Thibodeau SN, Marsoni S, Monges G, Labianca R, Kim GP, Yothers G, Allegra C, Moore MJ, Gallinger S and Sargent DJ. DNA mismatch repair status and colon cancer recurrence and survival in clinical trials of 5-fluorouracil-based adjuvant therapy. *J Natl Cancer Inst* 2011; 103: 863-75

Sleeman J, Moll J, Sherman L, Dall P, Pals ST, Ponta H and Herrlich P. The role of CD44 splice variants in human metastatic cancer. *Ciba Found Symp* 1995; 189: 142-51; discussion 151-6, 174-6

Snuderl M, Fazlollahi L, Le LP, Nitta M, Zhelyazkova BH, Davidson CJ, Akhavanfard S, Cahill DP, Aldape KD, Betensky RA, Louis DN and Iafrate AJ. Mosaic amplification of multiple receptor tyrosine kinase genes in glioblastoma. *Cancer Cell* 2011; 20: 810-7

Snover DC, Ahnen DJ, Burt RW, Odze RD: Serrated polyps of the colon and rectum and serrated polyposis *in* Bosman FT, Carneiro F, Hruban RH, Theise ND (eds): WHO Classification of Tumours of the Digestive System, 4th edition, International Agency for Research on Cancer, Lyon, 2010, pp 160-5

Spano JP and Vignot S. [EGF receptors in colorectal cancers]. *Bull Cancer* 2007; 94: F171-6

Steg AD, Bevis KS, Katre AA, Ziebarth A, Dobbin ZC, Alvarez RD, Zhang K, Conner M and Landen CN. Stem cell pathways contribute to clinical chemoresistance in ovarian cancer. *Clin Cancer Res* 2012; 18: 869-81

Stintzing S, Fischer von Weikersthal L, Decker T, Vehling-Kaiser U, Jäger E, Heintges T, Stoll C, Giessen C, Modest DP, Neumann J, Jung A, Kirchner T, Scheithauer W and Heinemann V. FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab as first-line treatment for patients with metastatic colorectal cancer-subgroup analysis of patients with KRAS: mutated tumours in the randomised German AIO study KRK-0306. *Ann Oncol* 2012; 23: 1693-9

Suzuki H, Masuda N, Shimura T, Araki K, Kobayashi T, Tsutsumi S, Asao T and Kuwano H. Nuclear beta-catenin expression at the invasive front and in the vessels predicts liver metastasis in colorectal carcinoma. *Anticancer Res* 2008; 28: 1821-30

Swanson RS, Compton CC, Stewart AK and Bland KI. The prognosis of T3N0 colon cancer is dependent on the number of lymph nodes examined. *Ann Surg Oncol* 2003; 10: 65-71

Talvinen K, Tuikkala J, Grönroos J, Huhtinen H, Kronqvist P, Aittokallio T, Nevalainen O, Hiekkanen H, Nevalainen T and Sundström J. Biochemical and clinical approaches in evaluating the prognosis of colon cancer. *Anticancer Res* 2006; 26: 4745-51

Tanaka H, Deng G, Matsuzaki K, Kakar S, Kim GE, Miura S, Sleisenger MH and Kim YS. BRAF mutation, CpG island methylator phenotype and microsatellite instability occur more frequently and concordantly in mucinous than non-mucinous colorectal cancer. *Int J Cancer* 2006; 118: 2765-71

Tanaka T. Colorectal carcinogenesis: Review of human and experimental animal studies. *J Carcinog* 2009; 8: 5

Tang XH and Gudas LJ. Retinoids, retinoic acid receptors, and cancer. *Annu Rev Pathol* 2011; 6: 345-64

Terzi C, Canda AE, Sagol O, Atila K, Sonmez D, Fuzun M, Gorken IB, Oztop I and Obuz F. Survivin, p53, and Ki-67 as predictors of histopathologic response in locally advanced rectal cancer treated with preoperative chemoradiotherapy. *Int J Colorectal Dis* 2008; 23: 37-45

Tong Y, Tan Y, Zhou C and Melmed S. Pituitary tumor transforming gene interacts with Sp1 to modulate G1/S cell phase transition. *Oncogene* 2007; 26: 5596-605

Tribler L, Jensen LT, Jørgensen K, Brünner N, Gelb MH, Nielsen HJ and Jensen SS. Increased expression and activity of group IIA and X secretory phospholipase A2 in peritumoral versus central colon carcinoma tissue. *Anticancer Res* 2007; 27: 3179-85

Tsukita S, Oishi K, Sato N, Sagara J and Kawai A. ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons. *J Cell Biol* 1994; 126: 391-401

Twelves C, Wong A, Nowacki MP, Abt M, Burris H, Carrato A, Cassidy J, Cervantes A, Fagerberg J, Georgoulias V, Husseini F, Jodrell D, Koralewski P, Kröning H, Maroun J, Marschner N, McKendrick J, Pawlicki M, Rosso R, Schüller J, Seitz JF, Stabuc B, Tujakowski J, Van Hazel G, Zaluski J and Scheithauer W. Capecitabine as adjuvant treatment for stage III colon cancer. *N Engl J Med* 2005; 352: 2696-704

Umar A, Boland CR, Terdiman JP, Syngal S, de la Chapelle A, Rüschoff J, Fishel R, Lindor NM, Burgart LJ, Hamelin R, Hamilton SR, Hiatt RA, Jass J, Lindblom A, Lynch HT, Peltomaki P, Ramsey SD, Rodriguez-Bigas MA, Vasen HF, Hawk ET, Barrett JC, Freedman AN and Srivastava S. Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. *J Natl Cancer Inst* 2004; 96: 261-8

Valentini V, Beets-Tan R, Borras JM, Krivokapić Z, Leer JW, Påhlman L, Rödel C, Schmoll HJ, Scott N, Velde CV and Verfaillie C. Evidence and research in rectal cancer. *Radiother Oncol* 2008; 87: 449-74

Van Cutsem E, Köhne CH, Láng I, Folprecht G, Nowacki MP, Cascinu S, Shchepotin I, Maurel J, Cunningham D, Tejpar S, Schlichting M, Zubel A, Celik I, Rougier P and Ciardiello F. Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. *J Clin Oncol* 2011; 29: 2011-9

van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, van der Horn K, Batlle E, Coudreuse D, Haramis AP, Tjon-Pon-Fong M, Moerer P, van den Born M, Soete G, Pals S, Eilers M, Medema R and Clevers H. The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* 2002; 111: 241-50

van Gijn W, Marijnen CA, Nagtegaal ID, Kranenbarg EM, Putter H, Wiggers T, Rutten HJ, Påhlman L, Glimelius B, van de Velde CJ and Group DCC. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer: 12-year follow-up of the multicentre, randomised controlled TME trial. *Lancet Oncol* 2011; 12: 575-82

Vasen HF, Watson P, Mecklin JP and Lynch HT. New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. *Gastroenterology* 1999; 116: 1453-6

Verhulst J, Ferdinande L, Demetter P and Ceelen W. Mucinous subtype as prognostic factor in colorectal cancer: a systematic review and meta-analysis. J Clin Pathol 2012; 65: 381-8

Vermeulen L, De Sousa E Melo F, van der Heijden M, Cameron K, de Jong JH, Borovski T, Tuynman JB, Todaro M, Merz C, Rodermond H, Sprick MR, Kemper K, Richel DJ, Stassi G and Medema JP. Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. *Nat Cell Biol* 2010; 12: 468-76

Vestermark LW, Jacobsen A, Qvortrup C, Hansen F, Bisgaard C, Baatrup G, Rasmussen P and Pfeiffer P. Long-term results of a phase II trial of high-dose radiotherapy (60 Gy) and UFT/l-leucovorin in patients with non-resectable locally advanced rectal cancer (LARC). *Acta Oncol* 2008; 47: 428-33

Vlotides G, Eigler T and Melmed S. Pituitary tumor-transforming gene: physiology and implications for tumorigenesis. *Endocr Rev* 2007; 28: 165-86

Vogler T, Kriegl L, Horst D, Engel J, Sagebiel S, Schäffauer AJ, Kirchner T and Jung A. The expression pattern of aldehyde dehydrogenase 1 (ALDH1) is an independent prognostic marker for low survival in colorectal tumors. *Exp Mol Pathol* 2012; 92: 111-7

Wakamatsu Y, Sakamoto N, Oo HZ, Naito Y, Uraoka N, Anami K, Sentani K, Oue N and Yasui W. Expression of cancer stem cell markers ALDH1, CD44 and CD133 in primary tumor and lymph node metastasis of gastric cancer. *Pathol Int* 2012; 62: 112-9

Walther A, Johnstone E, Swanton C, Midgley R, Tomlinson I and Kerr D. Genetic prognostic and predictive markers in colorectal cancer. *Nat Rev Cancer* 2009; 9: 489-99

Wang Y, Zhe H, Gao P, Zhang N, Li G and Qin J. Cancer stem cell marker ALDH1 expression is associated with lymph node metastasis and poor survival in esophageal squamous cell carcinoma: a study from high incidence area of northern China. *Dis Esophagus* 2012; 25: 560-5

Ward KC, Young JL, Gloecker Ries LA: Chapter 4: Cancers of the Colon and Rectum *in* Ries LAG, Young JL, Keel GE, Eisner MP, Lin YD, Horner M-J (eds): SEER Survival Monograph: Cancer Survival Among Adults: U.S. SEER Program, 1988-2001, Patient and Tumor Charasteristics. National Cancer Institute, SEER Program, NIH Pub. No. 07-6215, Bethesda, MD, 2007

Weinrauch Y, Elsbach P, Madsen LM, Foreman A, Weiss J. The potent anti-Staphylococcus aureus activity of a steril rabbit inflammatory fluid is due to a 14-kD phosholipase A2. *J Clin Invest* 1996; 97: 250-7

Weisenberger DJ, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, Kang GH, Widschwendter M, Weener D, Buchanan D, Koh H, Simms L, Barker M, Leggett B, Levine J, Kim M, French AJ, Thibodeau SN, Jass J, Haile R and Laird PW. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat Genet* 2006; 38: 787-93

Wendum D, Svrcek M, Rigau V, Boëlle PY, Sebbagh N, Parc R, Masliah J, Trugnan G and Fléjou JF. COX-2, inflammatory secreted PLA2, and cytoplasmic PLA2 protein expression in small bowel adenocarcinomas compared with colorectal adenocarcinomas. *Mod Pathol* 2003; 16: 130-6

West NP, Hohenberger W, Webe K, Perrakis A, Finan PJ and Quirke P. Complete mesocolic excision with central vascular ligation produces an oncologically superior specimen compared with standard surgery for carcinoma of the colon. *J Clin Oncol* 2010; 28: 272-8

Wielenga VJ, Heider KH, Offerhaus GJ, Adolf GR, van den Berg FM, Ponta H, Herrlich P and Pals ST. Expression of CD44 variant proteins in human colorectal cancer is related to tumor progression. *Cancer Res* 1993; 53: 4754-6

Wildes TM, Kallogjeri D, Powers B, Vlahiotis A, Mutch M, Spitznagel EL, Tan B and Piccirillo JF. The Benefit of Adjuvant Chemotherapy in Elderly Patients with Stage III Colorectal Cancer is Independent of Age and Comorbidity. *J Geriatr Oncol* 2010; 1: 48-56

Willett CG, Warland G, Coen J, Shellito PC and Compton CC. Rectal cancer: the influence of tumor proliferation on response to preoperative irradiation. *Int J Radiat Oncol Biol Phys* 1995; 32: 57-61

Wolin KY, Yan Y, Colditz GA and Lee IM. Physical activity and colon cancer prevention: a meta-analysis. *Br J Cancer* 2009; 100: 611-6

World Cancer Research Fund / American Institute for Cancer Research. Continuous Update Project summary. Food, Nutrition, Physical activity, and the Prevention of Colorectal Cancer 2011. Available at http://www.dietandcancerreport.org

Wu BW, Ma D, Li DF, Li YJ, Deng G, Zeng ZG, Zhang KJ, Zhang YH and Geng QS. [Expression of human pituitary tumor transforming gene 1 in colorectal cancer detected with real-time fluorescent quantitative PCR and its clinical significance]. *Ai Zheng* 2008; 27: 1217-21

Xing XF, Li H, Zhong XY, Zhang LH, Wang XH, Liu YQ, Jia SQ, Shi T, Niu ZJ, Peng Y, Du H, Zhang GG, Hu Y, Lu AP, Li JY, Chen S and Ji JF. Phospholipase A2 group IIA expression correlates with prolonged survival in gastric cancer. *Histopathology* 2011; 59: 198-206

Yang ZY, Shen WX, Hu XF, Zheng DY, Wu XY, Huang YF, Chen JZ, Mao C and Tang JL. EGFR gene copy number as a predictive biomarker for the treatment of metastatic colorectal cancer with anti-EGFR monoclonal antibodies: a meta-analysis. *J Hematol Oncol* 2012; 5: 52

Yao H, Ashihara E and Maekawa T. Targeting the Wnt/β-catenin signaling pathway in human cancers. *Expert Opin Ther Targets* 2011; 15: 873-87

Yoon CH, Kim MJ, Lee H, Kim RK, Lim EJ, Yoo KC, Lee GH, Cui YH, Oh YS, Gye MC, Lee YY, Park IC, An S, Hwang SG, Park MJ, Suh Y and Lee SJ. PTTG1 oncogene promotes tumor malignancy via epithelial to mesenchymal transition and expansion of cancer stem cell population. *J Biol Chem* 2012; 287: 19516-27

Yoshida A, Hsu LC and Davé V. Retinal oxidation activity and biological role of human cytosolic aldehyde dehydrogenase. *Enzyme* 1992; 46: 239-44

Yu R, Heaney AP, Lu W, Chen J and Melmed S. Pituitary tumor transforming gene causes aneuploidy and p53-dependent and p53-independent apoptosis. *J Biol Chem* 2000; 275: 36502-5

Zhang X, Horwitz GA, Prezant TR, Valentini A, Nakashima M, Bronstein MD and Melmed S.

Structure, expression, and function of human pituitary tumor-transforming gene (PTTG). *Mol Endocrinol* 1999; 13: 156-66

Zhu J, Xu Y, Gu W, Peng J, Cai G, Sun W, Shen W, Cai S and Zhang Z. Adjuvant therapy for T3N0 rectal cancer in the total mesorectal excision era-identification of the high risk patients. *Radiat Oncol* 2010; 5: 118

Zlobec I, Günthert U, Tornillo L, Iezzi G, Baumhoer D, Terracciano L and Lugli A. Systematic assessment of the prognostic impact of membranous CD44v6 protein expression in colorectal cancer. *Histopathology* 2009; 55: 564-75

Zlobec I, Vuong T, Compton CC, Lugli A, Michel RP, Hayashi S and Jass JR. Combined analysis of VEGF and EGFR predicts complete tumour response in rectal cancer treated with preoperative radiotherapy. *Br J Cancer* 2008; 98: 450-6

Zou H, McGarry TJ, Bernal T and Kirschner MW. Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. *Science* 1999; 285: 418-22

Ålgars A, Lintunen M, Carpén O, Ristamäki R and Sundström J. EGFR gene copy number assessment from areas with highest EGFR expression predicts response to anti-EGFR therapy in colorectal cancer. *Br J Cancer* 2011; 105: 255-62

Österlund P, Soveri LM, Isoniemi H, Poussa T, Alanko T and Bono P. Hypertension and overall survival in metastatic colorectal cancer patients treated with bevacizumab-containing chemotherapy. *Br J Cancer* 2011; 104: 599-604

Österlund P, Salminen T and Järvinen HJ. Suolistosyöpäpotilas terveyskeskuksessa. *Suomen Lääkärilehti*, 2012, 67: 1913-8