

# Contents

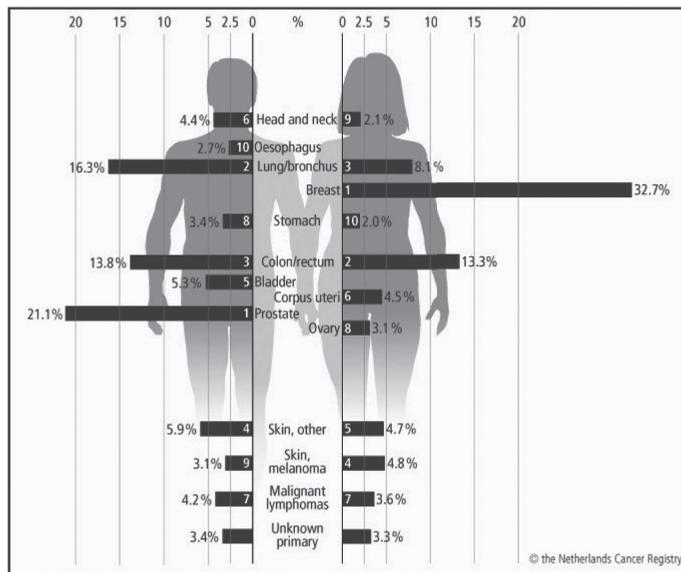
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## I. Epidemiology

Colorectal cancer (CRC) is one of the most common malignancies. In 2006, 412,900 new cases were diagnosed in Europe (12,9% of all cases of cancer) and 207,400 died of the disease [1]. CRC is associated with a Western lifestyle. The highest incidence rates occur in North America, Australia/New Zealand and Western Europe. Developing areas like Africa and Asia tend to have lower incidence rates. Epidemiological studies in migrant populations have demonstrated that environmental factors are the most important risk factors, more than genetic differences between populations [2].

In the Netherlands, colorectal cancer ranks third in men after prostate and lung cancer and second in women after breast cancer (Figure 1). In 2006, approximately 11.000 new individuals were diagnosed with colorectal cancer and with more than 4700 deaths it is the second most common cause of death due to cancer (the Netherlands Cancer Registry; <http://www.ikcnet.nl>). In the last two decades, incidence increased in men about 1% annually, in women this increase was much smaller [3]. Colorectal cancer is a disease of the elderly with a peak incidence around 60 to 74 years and the incidence of CRC is expected to rise as life expectancy of the general population increases. In the Netherlands, the cumulative life risk is about 5% by the age of 75 years.



**Figure 1.** Ranking of the ten most frequent invasive tumors among men and women in 2003 in the Netherlands (from: the Netherlands Cancer Registry).

## 2. Etiology

Colorectal cancer occurs most commonly sporadically and is inherited in 5% of cases [4,5]. In non-hereditary cases, individuals with a first degree relative with colorectal cancer have a 2 to 3-fold increased risk of developing this disease [6,7]. The cause of sporadic colorectal cancer is still not completely solved, but several environmental and lifestyle factors are believed to increase the risk of developing this disease.

### 2.1 Genetic factors

A number of hereditary colorectal cancer syndromes exist in which affected individuals carry genetic alterations in their germ-line cells. The most common inherited colorectal cancer syndromes are Familial Adenomatous Polyposis (FAP) and Lynch syndrome also known as Hereditary Non-Polyposis Colorectal Cancer (HNPCC).

FAP is an autosomal dominant inherited disorder, in which affected individuals develop hundreds to thousands of adenomas in their large intestine. These adenomas, often referred to simply as polyps, are precursor lesions of CRC. FAP patients often have a germ-line mutation of the *Adenomatous Polyposis Coli (APC)* gene. This mutation can initiate tumorigenesis, and patients develop polyps already during childhood or adolescence (5 to 15 years old) [4]. Malignant transformation invariably occurs in one or more of these adenomas and invasive cancer occurs before the age of 40 [4]. In addition to colorectal tumors, FAP patients can have tumors of the small intestine and stomach [8]. Attenuated FAP (AFAP) is a milder form of this disease with less than 100 colorectal adenomas and a later onset of disease.

Lynch syndrome is the most common hereditary cancer syndrome and it accounts for 1 to 5% of colorectal cancers [4]. Also, like FAP, this syndrome is an autosomal dominant disorder and patients develop colorectal cancer at a median age of 45 [8]. However, these patients lack a marked increase in the number of adenomas. Patients with Lynch syndrome inherit defects in one of the DNA mismatch repair genes *MLH1*, *MSH2*, *MSH6*, *PMS1* and *PMS2*, and these carriers have an 80% lifetime risk of developing colorectal cancer [6]. In contrast to FAP, the defect in Lynch syndrome primarily affects tumor progression. Tumors arising in these patients are genetically unstable, leading to an accumulation of mutations and consequently a rapid progression to cancer. The majority of the Lynch-associated carcinomas, approximately 70%, develop in the proximal colon, i.e. proximal to the splenic flexure [8]. In addition to colorectal cancer, these patients are at increased risk for cancers of at least seven other organs (endometrium, ovaries, stomach, small bowel, hepatobiliary epithelium, uroepithelial epithelium and brain) [8-10].

Recently, MYH-associated polyposis (MAP), an autosomal recessive syndrome, has been discovered, in which affected individuals inherit a germ-line mutation in the *MYH* gene at both alleles. The protein of this gene has a role in the base excision repair (BER) system. Patients with this disease also have multiple adenomas, but less than in FAP [11-13].

Other, more rare hereditary syndromes with an increased risk of colorectal cancer are juvenile polyposis (*SMAD4* and *BMPRII* mutation) Cowden- (*PTEN* mutation) and Peutz-Jeghers syndrome (*LKB1* mutation). These diseases are characterized by many hamartomatous polyps in the gastrointestinal tract [4,8,10].

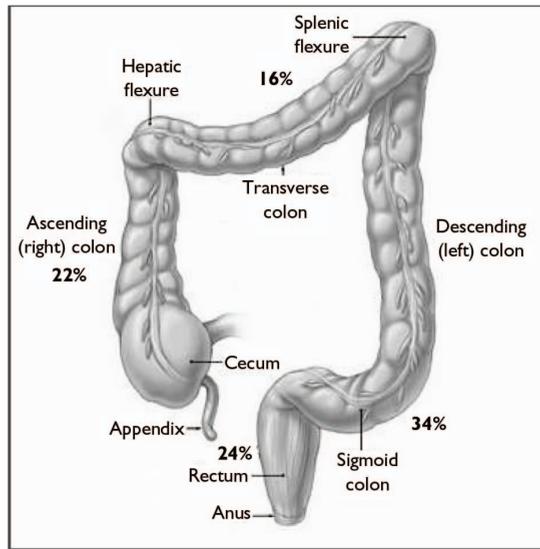
## 2.2 Environmental factors

Age is probably the single most important risk factor for colorectal cancer in the general population. Other conditions that predispose to the development of colorectal cancer include inflammatory bowel disease, i.e. ulcerative colitis and Crohn's disease [14,15]. The increased risk is related to the extent and duration of the disease, 2% by 10 years, 8% by 20 years and 18% by 30 years [16]. The importance of environmental factors in the pathogenesis of colorectal cancer is emphasized by the high incidence of the disease in the Western countries and among immigrants that moved from low-risk to high-risk regions. Associations of diet with CRC have been reported including positive correlations between colorectal cancer risk and a high intake of fat, red meat [17-19] and alcohol [20,21], as well as smoking [19,22-24], and inverse correlations have been reported with intake of fibers and/or vegetables [25], physical activity [26-28] and non-steroidal anti-inflammatory drugs (NSAIDs). However, several of these associations are still under debate [22, 29-31].

## 3. Pathology

Adenocarcinoma is the major histological type of colorectal cancer, which accounts for more than 90% of all malignancies of the large intestine. This thesis addresses these carcinomas, which originate from the epithelial lining of the crypts of the colorectal mucosa. Approximately 60% of CRCs arise in the distal part of the large intestine, i.e. distal to the splenic flexure (Figure 2), although the incidence of proximal CRCs seems to be rising [32,33].

Macroscopically, CRC typically has a polypoid or ulcerating appearance. Especially the proximal part of the large intestine, where the lumen has a large caliber, allows unhampered intraluminal growth. However, CRCs can also grow in an annular and constrictive way, giving a typical "apple core" configuration on barium enema examination. This growth pattern occurs more often in the distal part of the large intestine.



**Figure 2.** Anatomy of the large intestine. Percentages indicate the anatomical distribution of colorectal cancer.

Histologically, a number of sub-types can be discerned including mucinous and signet cell carcinoma. In addition, differentiation (i.e. the extent to which the growth pattern resembles that of the original colon mucosa) is graded as well, moderate and poor. The extent of tumor growth is staged according to the TNM classification of the American Joint Committee on Cancer (AJCC) and the International Union against Cancer (UICC). In this classification, the T refers to the degree of penetration of the tumor into the bowel wall, the N to the presence and number of lymph node metastases and the M to the presence of distant metastases. This classification was preceded by the Dukes classification from 1932, which has been widely used for a long time. Overtime this classification has seen numerous modifications, like the Astler-Coller classification, although the basic concept has remained unchanged (Table I). The TNM classification, in contrast to the Dukes or Astler-Coller staging system, includes both a clinical (pretreatment) and a pathological (post-surgical) classification. They are based on different methods of examination and serve different purposes [34,35]. In general, the cTNM is the basis for the choice of primary treatment and the pTNM is the basis for prognostic assessment and adjuvant treatment.

**Table I.** The different classification systems in colorectal cancer (From: [http:// www.oncoline.nl](http://www.oncoline.nl))

Dukes	Astler-Coller	TNM		Stage
<b>A</b>	A	Tis	N0 M0	0
	B1	T1-2	N0 M0	I
<b>B</b>	B2	T3	N0 M0	II
	B3	T4	N0 M0	
<b>C</b>	C1	T1-2	N+ M0	III
	C2	T3	N+ M0	
	C3	T4	N+ M0	
<b>D</b>	D	Tx	Nx M1	IV

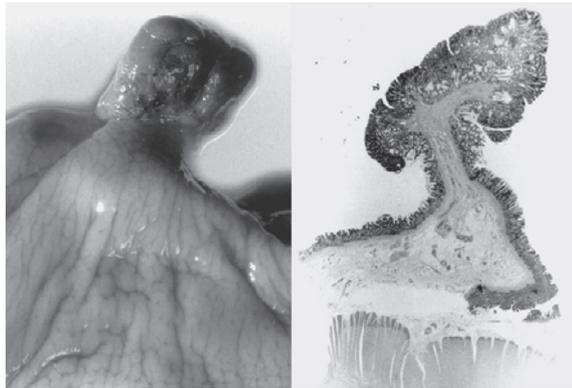
#### 4. Pathogenesis – Phenotype

The adenoma-carcinoma sequence, i.e. the theory that adenomas are the precursor lesions of all colorectal carcinomas, dominates already for many decades [36,37]. Adenomas arise from mucosal epithelium as a failure in the normal process of proliferation, differentiation and apoptosis. This failure leads to a shift of the proliferative compartment from the lower part to the upper part of the crypt, where the undifferentiated cells accumulate at the luminal surface and form a polyp. The macroscopic appearance of an adenoma varies from a pedunculated (Figure 3) to a sessile polyp. The term 'polyp' is commonly used for adenomas, but it is confusing as it is also used for other types of protrusions in the large intestine, which are no precursor lesions of adenocarcinoma. In addition, adenomas can also be flat.

Microscopically, adenomas exhibit varying degrees of epithelial dysplasia (i.e. mild, moderate or severe) revealed by hyperchromatic and polymorphic nuclei, nuclear crowding, loss of polarity and abnormal mitotic activity. These same phenotypical characteristics are also seen in carcinomas, but adenomas do not invade the surrounding tissue and do not have the potential to metastasize. On the basis of the epithelial architecture adenomas show tubular, villous or tubulovillous growth.

Adenomas are highly prevalent lesions occurring in more than 30% of the normal population over age 60 years. However, only about 5% of these adenomas ever progress to cancer [38]. The ability to distinguish adenomas that do progress to cancer from those that will not progress cannot be reliably made macroscopically. The risk of an adenoma to turn malignant is often based on three histopathological features: polyp size, villous architecture and severity of dysplasia. Although, villous and tubulovillous adenomas contain relatively more often foci of carcinoma than tubular adenomas, the prevalence in the later one is still high because tubular adenomas occur more often.

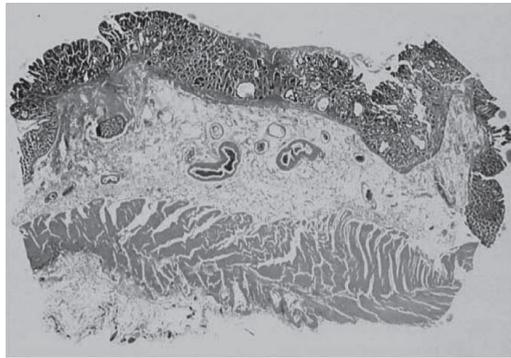
Yet, all this is based on cross sectional data, as no longitudinal data exist. These data will also never be generated, because the study design needed (i.e. leave adenomas in place until they become malignant) is unethical.



**Figure 3.** Penduculated colorectal adenoma, left the gross appearance and right the microscopic image.

Colorectal adenoma to carcinoma progression occurs gradually and takes many years. However, formal proof of the actual duration of this process is not available as adenomas are usually removed soon after their discovery. Studies in adenomatous polyposis (FAP) patients suggest that the evolution from an adenoma to an invasive carcinoma may take between 5 to 15 years [36].

The last decade, more evidence has accumulated that flat and serrated adenomas, next to polypoid adenomas, are two other precursor phenotypes of colorectal carcinomas [39-41]. Endoscopically, flat adenomas are flat or slightly elevated lesions, sometimes with a central depression, and often smaller than 1 cm in diameter (Figure 4). Compared with polypoid adenomas of similar size, flat adenomas show more frequently high-grade dysplasia and have a higher risk of invasion [40,42]. Serrated adenomas are histologically defined as adenomas that have serrated epithelium, like hyperplastic polyps, but that also contain cytological characteristics of conventional adenomas [43]. Serrated adenomas are frequently localized in the proximal part of the large intestine.



**Figure 4.** Microscopic example of a flat adenoma.

## 5. Pathogenesis – Genotype

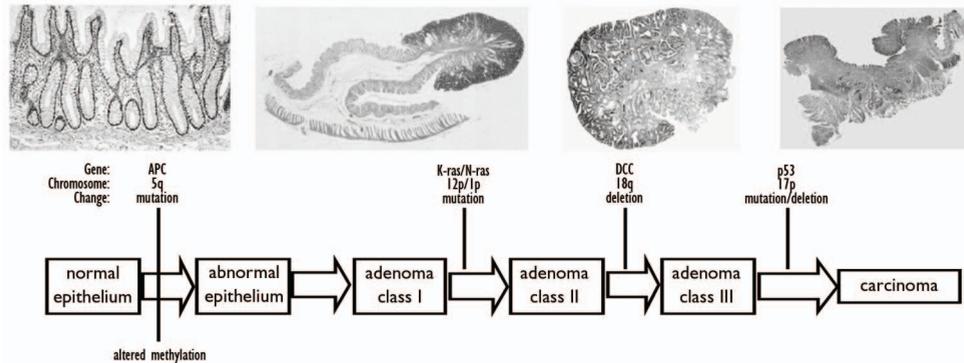
The multistep process of colorectal carcinogenesis, from normal epithelium through adenoma to malignant neoplasia, is accompanied by a progressive accumulation of genetic and epigenetic alterations. Alterations in the genome can lead to activation of genes which result in 'gain of function' and cause tumor formation, so-called oncogenes, or to inactivation of genes that inhibit tumor formation, so-called tumor suppressor genes. Oncogenes are genes that encode a normal protein that is expressed at inappropriately high levels or are mutated genes that exhibit inappropriately high activity, both cases stimulating tumor growth. Mechanisms that can lead to oncogene activation include point mutation, chromosomal translocation and amplification. Tumor suppressor genes are genes, which promote tumor growth by loss of function. In 1971, Knudson proposed that for loss of function of a tumor suppressor gene, both alleles of that specific gene need to be inactivated, the "two hit model" [44]. Mechanisms that can inactivate tumor suppressor genes include, next to point mutations, deletions and promoter hypermethylation.

### 5.1 Genetic instability

The first genetic model of colorectal carcinogenesis was proposed by Fearon and Vogelstein in 1990 [45], and emphasizes the role of mutation or loss of heterozygosity of specific tumor suppressor genes, i.e. *APC*, *DCC* and *p53*, and the mutation of the *KRAS* oncogene (Figure 6).

The transformation of normal epithelium to a malignant invasive tumor involves, however, multiple biological processes, i.e. cell proliferation, apoptosis, immortalization, angiogenesis, invasion and metastasis [46]. The deregulation of these processes requires multiple genetic changes, which lead to activation or inactivation of specific biological pathways. A genetically unstable environment that

occurs early in tumorigenesis is a condition for adenoma to carcinoma progression [47,48]. The two best recognized forms of genetic instability in colorectal cancer are chromosomal instability (CIN) and microsatellite instability (MSI) [49].



**Figure 6.** Genetic changes in the multistep model of colorectal carcinogenesis. (Adapted from Fearon and Vogelstein [45])

### 5.1.1 Chromosomal instability

Chromosomal instability is the most common type of genomic instability in solid tumors. It occurs in approximately 85% of colorectal carcinomas and is characterized by the accumulation of numerical and/or structural chromosomal abnormalities (aneuploidy). The mechanisms that induce chromosomal instability are only partly understood. The cause of CIN is thought to be a defect in the regulation of DNA replication checkpoints and in processes that control chromosome segregation during mitosis, i.e. mitotic-spindle checkpoints [50-52].

In the past, it was believed that gross numerical and structural chromosome alterations in invasive cancer were merely a side-effect of genetic instability driven by preceding mutations in oncogenes and tumor suppressor genes. This idea has maintained for a long time mainly because data on chromosomal alterations in early, pre-invasive tumors were scarce. This has changed with the introduction of Comparative Genomic Hybridization (CGH) as a new chromosome analysis technique in 1992 [53]. The identification of specific patterns of chromosome gains and losses that occur during the adenoma-carcinoma sequence and the demonstration that CIN is an early event in tumor formation that increases with tumor progression is consistent with the idea that CIN is pathogenetic in colorectal cancer [49,54,55]. DNA copy number alterations can lead to deregulation of gene expression, which may contribute to the development or progression of

cancer [56-61]. In the last decade, considerable amount of (array)CGH studies have substantially increased and demonstrated the occurrence of many chromosomal alterations in colorectal carcinogenesis, such as losses of chromosomes 1p, 4q, 5q, 8p, 14q, 15q, 17p, 18p, 18q, 21q and 22q, and gain of chromosomes 1q, 7p, 7q, 8q, 11q, 12p, 13q, 16p, 16q, 19q, 20p and 20q [62-83].

### 5.1.2 Microsatellite instability

In contrast to chromosomal instability, tumors that display microsatellite instability (MSI) are often diploid or near-diploid at the chromosomal level and harbor frequent alterations in microsatellites, which are short repetitive nucleotide sequences. This form of genetic instability arises when there is a defect in the DNA mismatch repair (MMR) system. The MMR system consists of a complex of proteins, i.e. MLH1, MSH2, MSH6, PMS1 and PMS2, which recognize and repair base pair mismatches that occur during DNA replication, due to DNA polymerase slippage in microsatellites. When this system fails, increased mutations in microsatellites located throughout the genome occur [84] and if these mutations occur in the coding region of a specific gene, this may affect the expression and/or function of that gene.

Microsatellite instability is observed in almost all colorectal carcinomas from patients with Lynch syndrome and occurs in 15% of sporadic colorectal cancer [85]. Lynch syndrome patients harbor germline mutations in one of the MMR genes, with more than 90% of the cases involving *MLH1* or *MSH2*. In sporadic colorectal cancer inactivation of the MMR system can, next to a somatic mutation in one of the MMR genes, also be induced by promoter hypermethylation of the *MLH1* gene.

Carcinomas that arise via the MSI pathway do show some distinctive phenotypic features, including proximal location, poor or mucinous differentiation, lymphocyte infiltration and, in general, these tumors have a better prognosis [86,87].

### 5.1.3 DNA mutations

Next to copy number alterations and a deficient MMR system, gain or loss of function of a gene can also be induced by spontaneous (somatic) mutations in the DNA sequence. A point mutation, a single nucleotide base substitution that results in a structurally altered protein (missense mutation) can give proteins that e.g. have a higher affinity for their ligand, become constitutively active, or gain less susceptibility for degradation. Point mutations can also lead to a premature stopcodon, which results in a partly transcribed protein (truncated) that is often non-functional (nonsense mutations). Frameshift mutations, insertion or deletion of base pairs, can disrupt the reading frame of a DNA strand, also resulting in a premature stopcodon or the production of an altered protein.

## 5.2 Epigenetics

Next to genetic alterations, epigenetic changes also play a role in colorectal carcinogenesis [88]. Epigenetic mechanisms, like DNA methylation and histone modification, influence gene expression without altering the DNA sequence or copy number. DNA methylation is present throughout the genome and plays a critical role in the control of gene activity and the architecture of the nucleus of the cell [89]. In humans, approximately 70% of CpG dinucleotides, i.e. a cytosine that precedes a guanosine in the DNA sequence, are methylated (a methyl group is added to the cytosine). Tumor cells show global hypomethylation in comparison to their normal counterpart [90], which may be associated with chromosomal instability [91]. Regions that are enriched for CpG dinucleotides are called CpG islands. They are frequently present in the promoter region of genes and are normally maintained in an unmethylated state. In tumors, many of these CpG islands become hypermethylated, which can cause transcriptional repression and thereby downregulation of gene expression [92,93].

Acetylation and methylation of histones, i.e. proteins around which the DNA is wrapped, have direct effect on gene transcription. Generally, histone acetylation is associated with transcriptional activation and deacetylation with repression, but the effect of histone methylation depends on the type of amino acid and its position in the histone tail [89].

## 5.3 Critical pathways in colorectal cancer

Colorectal carcinogenesis is characterized by the accumulation of (epi)genetic alterations, affecting several genes related to several different pathways, like Wnt, RAS, phosphoinositide3-kinase (PI3K), transforming growth factor  $\beta$  (TGF $\beta$ ) and p53. These pathways cross-talk at different levels [94].

### Wnt pathway

The Wnt/ $\beta$ -catenin/TCF signaling pathway is essential for controlling intestinal epithelial cell proliferation. In normal cells, free  $\beta$ -catenin binds to a complex consisting of APC, Axin and glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ), which targets  $\beta$ -catenin for degradation via ubiquitination. Loss of APC function results in constitutively active Wnt signaling, because the degradation complex is unable to bind to  $\beta$ -catenin, resulting in intracellular free  $\beta$ -catenin accumulation and translocation to the nucleus where it drives transcription of multiple genes via T-cell factor (TCF)/ lymphoid enhancer factor (LEF) transcription factor family [95-98]. The APC gene is located on chromosome 5q21 and inactivation of both APC alleles can be achieved by mutation, allelic loss (LOH) or promoter hypermethylation [99,100]. Mutations are observed in up to 80% of sporadic colorectal carcinomas and are found in a similar frequency in adenomas [43,95,101-103]. APC mutations are characteristically identified in the earliest adenomas, indicating that this gene plays a critical role

as the “gatekeeper” in the multistep progression from normal epithelial cell to cancer cell. Loss of functional APC has also been suggested to interfere with the normal regulation of mitosis, contributing to chromosomal instability [98,104].

In tumors with wild-type APC, a  $\beta$ -catenin stabilizing mutation [96,103,105,106] or an inactivating AXIN2 mutation [107] can provide an alternative mechanism for Wnt pathway dysregulation. Among the target genes of the Wnt signaling pathway are *c-MYC*, *cyclin D1*, *matrix metalloproteinase-7 (MMP-7)* and *immunoglobulin transcription factor 2 (ITF-2)* [97,98]. Constitutively active Wnt signaling in colorectal carcinogenesis leads to upregulation of these genes [97].

### **RAS and PI3K pathways**

The RAS and PI3K signaling pathways are strongly interconnected and play an important role in tumorigenesis [108]. RAS and PI3K are downstream targets of receptor tyrosine kinases (RTKs), such as epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR) and insulin-like growth factor-I receptor (IGF1R), which become activated when the receptor binds to its ligand.

When activated, RAS recruits the serine/threonine kinase RAF that phosphorylates the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinases 1 and 2. Activated MAPKs are imported into the nucleus where they phosphorylate specific transcription factors involved in cell growth, differentiation and survival [108,109]. RAS plays a minor role in normal signaling to PI3K, but oncogenic RAS is a potent activator of PI3K [94,110].

The KRAS oncogene encodes a GTP-binding protein at the inner surface of the plasma membrane that transduces extracellular signals. KRAS mutations cause loss of inherent GTPase activity, which leads to constitutive signaling [110]. KRAS is the most commonly mutated gene in the RAS/RAF/MAPK and PI3K/Akt pathways and activating mutations are found in approximately 40% of colorectal carcinomas [101,102,108,111]. KRAS mutations have been identified in similar frequency in carcinomas and adenomas larger than 1 cm [101,102]. In contrast, smaller adenomas have much lower prevalence of KRAS mutations.

Mutations of the BRAF oncogene, which is a member of the RAF family, are less common in CRC. In sporadic CRCs with the CIN phenotype, BRAF mutations are rarely observed (<15%) [111-114], they are much more frequent in CRCs displaying the MSI phenotype (30% to 63%) [111, 114]. In CRC, BRAF mutations are inversely correlated with KRAS mutations [114].

Activated PI3K phosphorylates the protein serine/threonine kinase Akt, which lead to cell growth, apoptosis resistance, invasion and migration [109]. Phosphatase and tensin homologue (PTEN) is a tumor suppressor protein that inhibits the PI3K/Akt signaling pathway. Activating mutations of the PIK3CA oncogene, which encodes the p110 $\alpha$  catalytic subunit of the PI3K protein, and inactivating

mutations of *PTEN* have been described in ~20% [111, 115, 116] and ~10% [117, 118] of colorectal carcinomas, respectively.

### **TGF $\beta$ pathway**

The TGF $\beta$  family is involved in the regulation of many cellular processes [119]. The intracellular effectors of TGF $\beta$  signaling, the Smad proteins, are activated by TGF $\beta$  receptors and translocated to the nucleus, where they regulate transcription of their target genes [120, 121]. In epithelial cells, the TGF $\beta$  signaling pathway has a growth inhibitory effect on proliferation by inducing expression of the cyclin kinase inhibitors p15INK4b, p21CIP1 and p27KIP1 which block cyclin and cyclin-dependent kinases from phosphorylating the retinoblastoma protein (pRb) [119]. Colorectal tumorigenesis is accompanied by alterations of the TGF $\beta$  signal transduction pathway. In 20% of colorectal carcinomas and in 5% of non-invasive tumors inactivating *SMAD4* mutations have been found [119, 122, 123]. Also allelic loss of 18q, where *SMAD4* is located, is a common event and has been identified in ~70% of primary colorectal cancers [102, 122] and in ~60% of advanced adenomas, however, this event is rare in early adenomas (10% to 30%) [102]. Inactivating *SMAD2* mutations are found in 6% of CRC [123]. In 90% of the tumors with deficient MMR system, mutations in microsatellites in the coding region of the TGF $\beta$  type II receptor gene (*TGF $\beta$ RII*) occur, leading to its inactivation [124, 125].

### **p53 pathway**

The p53 pathway plays a key role in cell cycle regulation, programmed cell death (apoptosis) and is important in maintaining DNA integrity [126]. The p53 network is activated when cells are stressed or damaged, inhibiting progress through the cell cycle followed by senescence, apoptosis and sometimes repair [127]. This pathway can be triggered by DNA damage through ATM/ATR and Chk1/Chk2 kinases, or by oncogenic signaling (i.e. c-MYC and E2F) through the p53-stabilizing protein p14<sup>ARF</sup> [126, 128]. When p53 is activated it binds to specific DNA sequences and activates the transcription of adjacent genes, including various pro-apoptotic Bcl2 family members (i.e. *PUMA*, *NOXA*, *BID* and *BAX*), as well as components of death-receptor signaling (i.e. *FAS*) and the apoptotic-effector machinery (i.e. *APAF1* and *PIDD*) [127, 129]. The tumor suppressor gene p53 is located on chromosome 17p13.1, and is the gene most frequently found mutated in solid tumors [128]. Inactivating point mutations of p53 or allelic loss of 17p have been identified in 50 to 75% of sporadic colorectal cancers [101, 130, 131] while these are found in only a small subset of adenomas (4% to 26%) [102]. Thus, genetic alteration of p53 is a late event in the adenoma-carcinoma sequence. Whereas CIN-positive tumors show frequent p53 mutation, MSI-positive tumors do not. These tumors instead, harbor in 50% of the cases inactivating *BAX* mutations [105, 132].

## 6. Clinical needs in colorectal cancer

### 6.1 Identification of high risk adenomas

Despite advances in treatment, overall mortality rates of colorectal cancer have remained relatively unchanged and 5-year survival is 59% (the Netherlands Cancer Registry; <http://www.ikcnet.nl>). This is due to the fact that at time of diagnosis, in most instances the tumor is already at advanced stage of the disease. Survival can be improved with secondary prevention, i.e. detection and removal of the disease in an early or even premalignant stage. For the detection of colorectal tumors, screening by endoscopy (colonoscopy or sigmoidoscopy) is the 'gold standard', which has the advantage that polypectomy and/or biopsies can be performed during the same procedure.

Adenomas can be adequately treated by endoscopic polypectomy [133] because these tumors do not show invasion in the surrounding tissue and do not have the potential to metastasize. Therefore, colorectal tumors are preferably removed in this premalignant stage. Theoretically, the removal of all adenomas could reduce the incidence of colorectal cancer to zero. However, only around 5% of all these adenomas will ever progress to cancer [38]. Therefore, the ability to distinguish these adenomas with a high risk of progression towards carcinoma from adenomas that will not progress is highly relevant for colorectal cancer screening, as this difference cannot be made macroscopically. Thus, there is a need to identify markers capable of discriminating 'high risk' adenomas from 'low risk' adenomas.

### 6.2 Risk assessment in primary colorectal cancer

At the moment, the most important prognostic factor in colorectal cancer is the tumor stage at time of diagnosis. Large variations exist in patient survival rates (Table 2), with survival of ~90% for localized cancer and ~40% for tumors that have already metastasized (the Netherlands Cancer Registry; <http://www.ikcnet.nl>).

Obviously these survival data imply standard therapy, which includes surgery, often combined with (neo)adjuvant therapies [134]. Surgery is the principal treatment for TNM stage I-III colorectal cancer and is curative in approximately 40 to 50% of patients. Adjuvant chemotherapy is given to colon cancer patients if lymph node metastases (stage III) are present. Initially, therapy consisted of 5-fluorouracil (5-FU) plus leucovorin (LV). The combination of a fluoropyrimidine, or the oral fluoropyrimidine capecitabine as a useful alternative to intravenous 5-FU, with oxaliplatin further increased clinical outcome [135,136]. Although the benefit of adjuvant chemotherapy for stage III colon cancer is acknowledged, the use of adjuvant chemotherapy in patients with stage II colon cancer is still under investigation [137-139]. Patients with stage II tumors constitute a particularly

heterogeneous population and not all patients are likely to benefit from adjuvant chemotherapy. A decision to treat stage II colon cancer is often made on the basis of a ‘high risk’ profile; T4 stage, perforation, poorly differentiated histology, venous invasion or inadequately sampled lymph nodes [137,140]. In rectal cancer, neoadjuvant radiotherapy with or without chemotherapy, has proven to reduce local recurrence rates, and followed by total mesorectal excision (TME) is the treatment of choice [141]. The value of adjuvant chemotherapy is still under investigation [142,143].

**Table 2.** Correlation between TNM stage and survival in colorectal cancer in the Netherlands (From: the Netherlands Cancer Registry; <http://www.ikcnet.nl>).

TNM stage	5-year survival (%)
Stage I	>90
Stage II	78
Stage III	61
Stage IV	<10

Colorectal cancer is biologically a heterogeneous disease, which leads to differences in clinical behavior, including the risk of metastasis. Therefore, the TNM classification on its own may be insufficient to predict clinical outcome and biomolecular features of CRC may have additional prognostic value.

### 6.3 Prediction of response to systemic therapy in advanced colorectal cancer

In patients with distant metastases there are no curative treatment options, but patients can have improved quality of life and longer survival with chemotherapy. For decades, 5-FU/LV has been the standard therapy. Median overall survival times have increased by the use of new cytotoxic drugs, like irinotecan and oxaliplatin. Furthermore, the oral fluoropyrimidine prodrug capecitabine has been proven a useful alternative to intravenous 5-FU. The development of new targeted drugs, such as the vascular endothelial growth factor (VEGF) – and epidermal growth factor receptor (EGFR)-antibodies, have further improved clinical outcome in patients with advanced disease.

With these new therapeutic options, management of advanced CRC has become increasingly complex and treatment protocols are still largely based on a “one size fits all” approach, despite the fact that only a subset of patients will respond. In this context, biomolecular features of colorectal tumors become increasingly important as predictors of response to therapy, like KRAS status in

the case of anti-EGFR therapy. Given the high cost and evident side effects of these new therapies, there is an increasing need for such biomarkers.

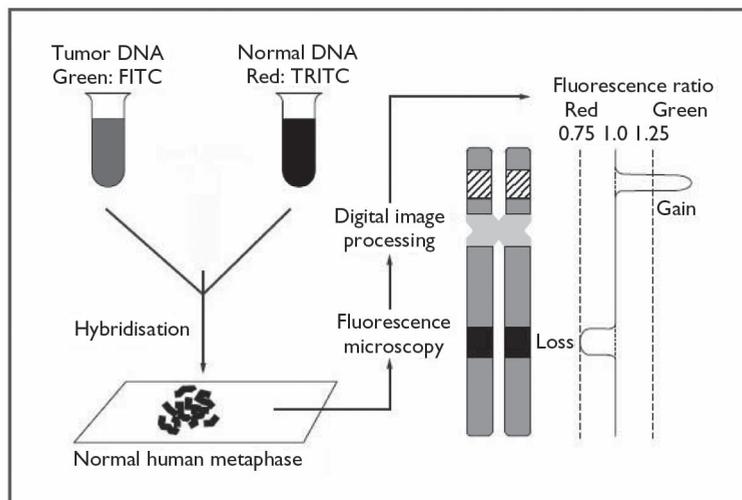
## 7. Tumor profiling tools

### 7.1 Comparative genomic hybridization

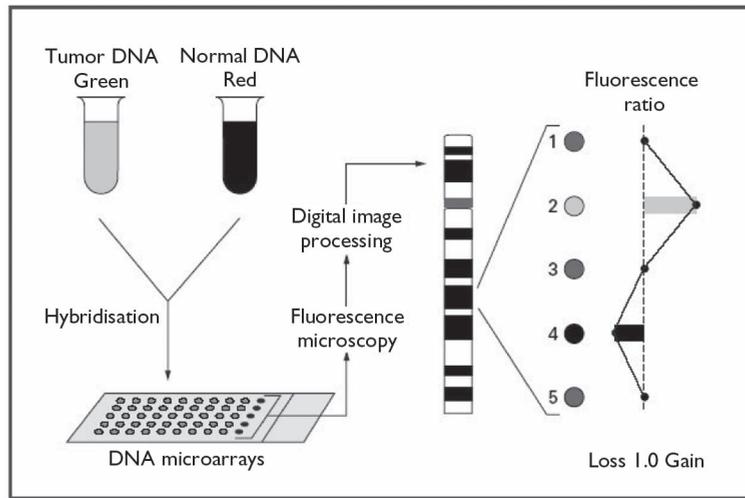
Chromosome-based Comparative Genomic Hybridization, which was first described by Kallioniemi [53], is a technique which has been widely used to analyze DNA copy number alterations, in a single experiment, on a genome-wide scale [144,145]. This method has overcome many of the drawbacks of conventional cytogenetic analysis of solid tumors, as it avoids the need for tissue culture and does not require the technically demanding analysis of complex karyotypes. Tumor and normal DNA are differentially labeled with fluorochromes (i.e. green and red labels, respectively) and hybridized to normal metaphase preparations. The labeled tumor and normal DNAs compete for hybridization to the chromosomes. Using fluorescence microscopy and digital image analysis, the green to red fluorescence ratio along all chromosomes is quantified. A gain or amplification in the tumor genome will be visualized by an excess of the green signal (ratio  $>1.0$ ), whereas a deletion or loss in the tumor genome is seen by an excess of red signal (ratio  $<1.0$ ) (Figure 7).

However, one of the limitations of chromosome-based CGH is the limited resolution for detecting DNA copy number alterations of  $\sim 10$  Mb. As a consequence, small alterations are frequently missed and the genomic boundaries of the alterations are not very well defined, limiting the correlation of specific genes to a specific alteration. This problem has largely been solved by the introduction of (micro)array-based CGH in 1997 [146,147], which resulted in increased sensitivity and much higher resolution (Figure 8). With this method, based on the same principle as chromosome-based CGH, an array of spotted genomic DNA probes of known origins serves as hybridization target. The resolution of array-based CGH is determined by the size of the DNA probe used to construct the array and the density with which they are selected from the genome. In the beginning, targets were obtained from large-insert genomic clones, such as BAC (Bacterial Artificial Chromosome) or PAC (P1-derived Artificial Chromosome) clones and the average resolution consisted of  $\sim 1$  Mb [148]. This was followed by tiling resolution arrays consisting of  $\sim 30,000$  overlapping BAC clones, covering the entire human genome [149,150]. Further improvement was achieved by using arrays of spotted oligonucleotides instead of the relatively large BAC and PAC clones (150-200 kb) [150-153]. Nowadays, the options for detecting DNA copy number alterations on a genome-wide basis and base-pair resolution are developing rapidly. CGH arrays can have resolutions of up to

2 million oligonucleotides per array, allowing the detection of focal deletions and amplifications, which facilitates the identification of genes affected by the chromosomal alteration. Several oligoarray platforms are commercially available, Affymetrix, Agilent, Illumina and NimbleGen, with different labeling and hybridization methods [154]. Some of these platforms include, next to the oligonucleotides for the detection of copy number alterations, probes for the detection of single nucleotide polymorphisms (SNPs), which provide information on loss of heterozygosity (LOH) or allelic imbalance.



**Figure 7.** Schematic overview of chromosome-based CGH. Tumor and normal DNA are labeled with green and red fluorochromes, respectively, and hybridized to normal metaphases. Images of the fluorescent signals are captured and the green to red ratios are digitally quantified for each chromosomal locus along the chromosomal axis.



**Figure 8.** Schematic overview array-based CGH. Tumor and normal DNA are labeled with green and red fluorochromes, respectively, and hybridized to genomic DNA attached to a glass slide. Images of the fluorescent signals are captured and the green to red ratios are digitally quantified for each target.

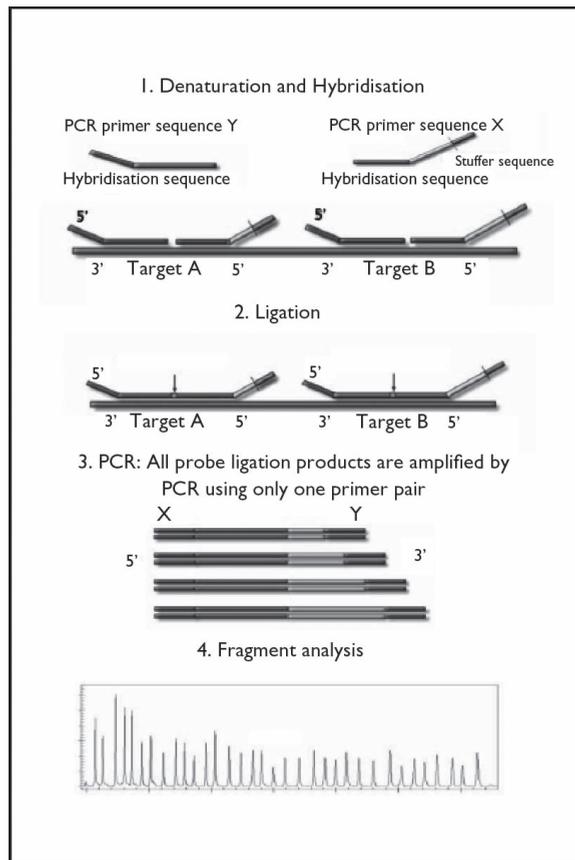
## 7.2 Multiplex Ligation-dependent Probe Amplification

Multiplex Ligation-dependent Probe Amplification (MLPA) is a quantitative multiplex PCR-based approach that allows to determine the relative DNA copy number of up to 40 individual genes, in a single experiment, requiring only minimal amounts of DNA [155]. In MLPA, each probe is made up of two hemiprobes consisting of a synthetic and a M13 derived oligonucleotide which contain target-specific sequences. The hemiprobes are designed to hybridize immediately next to each other. Only after hybridization they can be ligated to form one strand, which can be amplified by PCR. The ligated probes for different genes in a probe set have the same end sequences so they can be amplified with a universal primer. The amount of PCR product is proportional to the amount of target present in the sample. Since all probes contain a stuffer sequence, the length of which is different for every probe in a set, PCR products can be sorted by capillary electrophoresis, allowing the measurement of relative DNA copy number status of multiple individual target genes in a single experiment.

## 7.3 Expression microarrays

Combining gene expression data with DNA copy number data allows to measure gene dosage effect and identification of pivotal tumor-related genes. Differential gene expression in tumors can also be determined by microarray-based techniques [156]. As with array-based CGH, mRNA

expression of thousands of genes can be screened in a single experiment. Fluorescent labeled tumor RNA (or complementary DNA) and normal RNA are hybridized on arrays containing either complementary DNA (cDNA) or oligonucleotide sequences representing specific genes. After hybridization, the fluorescence ratio of every spot on the array is quantified, indicating the relative expression level of the associated gene within a given sample.



**Figure 9.** Schematic overview of the MLPA® technique. After hybridisation to their target sequence in the sample DNA, the probe oligonucleotides are enzymatically ligated. One probe oligonucleotide contains a non-hybridising stuffer sequence of variable length. Ligation products can be amplified using PCR primer sequences X and Y. Amplification products of each probe have a unique length. Amplification products are separated by electrophoresis. Relative amounts of probe amplification products, as compared to a reference DNA sample, reflect the relative copy number of target sequences (from: <http://www.mlpa.com>).

## 8. Aims and outline of this thesis

### 8.1 Aims

At time of diagnosis, most colorectal cancer patients already have late stage disease. Despite advances in treatment, overall mortality rates of colorectal cancer have remained relatively unchanged. Colorectal cancer is heterogeneous with respect to many clinical, pathological and biological features, therefore, traditional histopathologic staging may be insufficient in predicting clinical outcome. Improved treatment outcomes based on individualized treatment protocols, requires a better understanding of the associations between CRC biology and clinical phenotype. Pathogenesis of colorectal cancer is driven by an accumulation of (epi)genetic events that to a large extent occur at the chromosomal level and more insight into these alterations may contribute to a better understanding of colorectal carcinogenesis. The aims of the present thesis were to investigate DNA copy number changes in colorectal cancer and its precursor lesions, and to identify potential prognostic and predictive markers.

### 8.2 Outline

The study in **Chapter 2** aims to identify genetic alterations capable of discriminating adenomas with a high risk of progression towards carcinoma, from adenomas that will not progress, and to refine the current genetic models of colorectal adenoma to carcinoma progression. To this end, a large series of colorectal tumors in various stages of development are investigated by both genome-wide analysis by chromosome-based CGH as well as mutation analysis of genes involved in colorectal carcinogenesis.

Next to genetic alterations, the adenoma-carcinoma sequence is also driven by epigenetic changes. In **chapter 3** we investigated the association of epigenetic events with genetic events in colorectal cancer development using the same dataset as in chapter 2.

Next to the polypoid adenomas, two other CRC precursor phenotypes have been reported, i.e. flat and serrated adenomas. It has been proposed that these different phenotypes could be associated with different biological pathways leading to colorectal cancer. Therefore, in **chapter 4** a series of flat adenomas and carcinomas were analyzed at the chromosomal level with chromosome-based CGH and MLPA, and compared to their polypoid counterpart.

Risk assessment in primary colorectal cancer is largely based on tumor stage. However, colorectal cancer is biologically a heterogeneous disease, which leads to differences in clinical behavior, including the risk of metastasis. Adjuvant chemotherapy has improved outcome of patients with stage III colon cancer. Although, controversy exists if patients with stage II carcinomas should be treated with adjuvant chemotherapy, a subset of these patients eventually develops metastases

and would probably benefit from adjuvant treatment. To identify these poor prognosis patients, additional markers are needed. **Chapter 5** describes a pilot study in which chromosomal alterations are related to patient's survival.

Gain of the long arm of chromosome 20 is one of the most common chromosomal alterations in colorectal carcinogenesis (chapter 2) and also frequently occurs in flat adenomas (chapter 4). This genetic alteration also has been found to be related to worse clinical outcome in colorectal cancer patients (chapter 5). So, these findings suggest that chromosome 20q harbors oncogenes that are particularly relevant to colorectal cancer. In **chapter 6** putative oncogenes at 20q involved in colorectal adenoma to carcinoma progression were investigated by measuring gene dosage effects by means of array-based CGH and microarray expression analysis.

In the last stage of colorectal cancer, i.e. when metastatic disease is present, a clear need exists for identifying predictors of response to systemic therapy. To this end, in **chapter 7** we analyzed if genome-wide DNA copy number profiles of individual tumors hold information that can predict response to combined capecitabine and irinotecan therapy in advanced colorectal cancer.

Along the same lines, we next investigated whether DNA copy number profiles of CRC hold information additional to *KRAS* mutation status. Mutated *KRAS* is a powerful predictive marker to discriminate advanced CRC patients that will or will not respond to EGFR targeted therapy. CRC patients with *KRAS* mutations do not respond to anti-EGFR therapy. However, response rates in colorectal cancer with wild-type *KRAS* are also low, indicating that other factors, next to *KRAS* mutation status, influence the response to EGFR inhibitors. In **chapter 8** we investigated if differences in DNA copy number profiles in colorectal carcinomas with or without *KRAS* mutation occur and if we could identify different subgroups within the wild-type *KRAS* carcinomas.

Finally, in **chapter 9** all studies in this thesis are summarized and discussed.

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