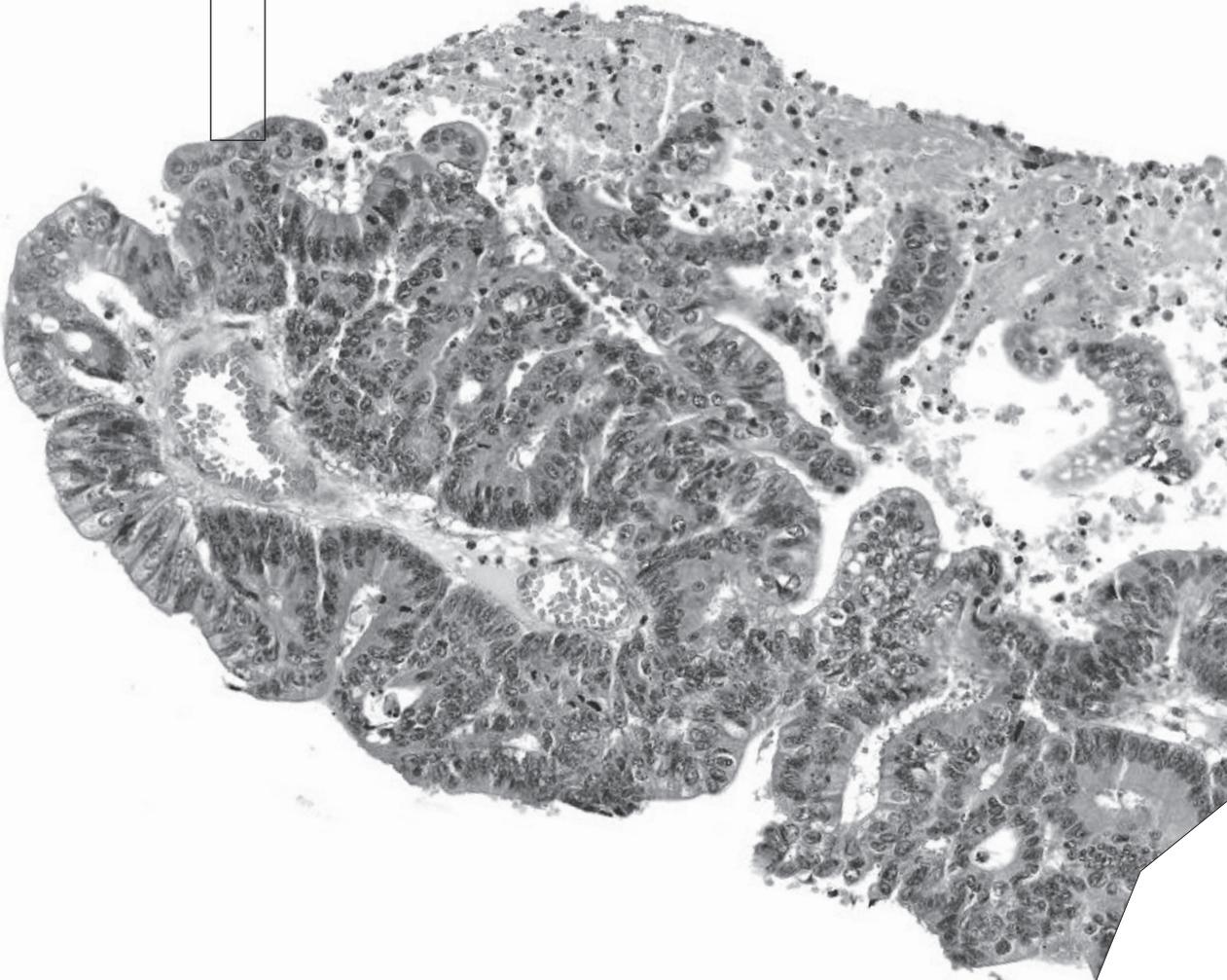
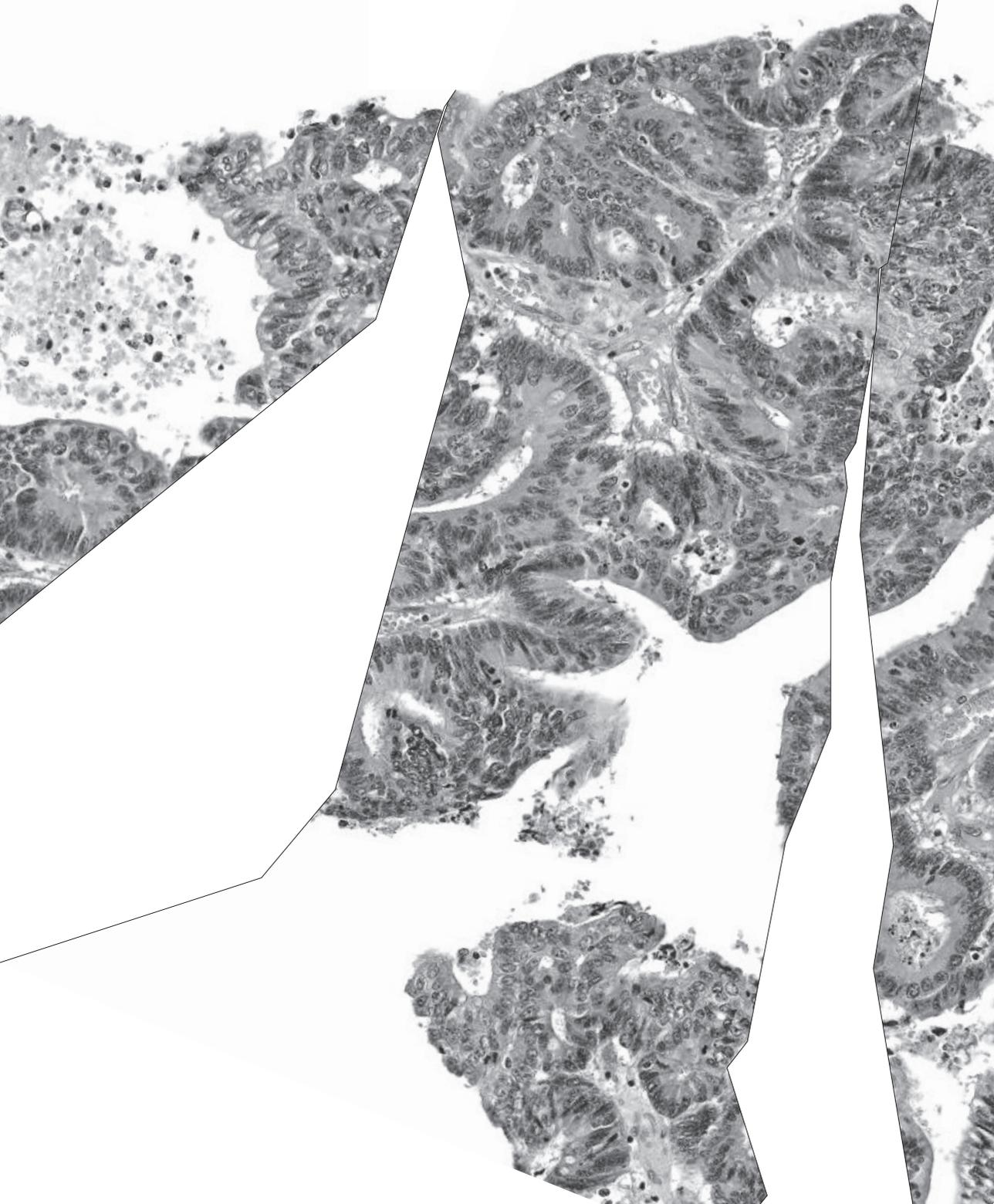


1



General introduction



DIAGNOSIS AND TREATMENT OF COLORECTAL CANCER

Colorectal cancer (CRC) is a major health care problem and one of the leading contributors to cancer mortality with a worldwide incidence of over 1.3 million cases per year [1]. Overall, about 50% of all CRC patients will eventually die due to disease progression, which makes it the fourth leading cause of cancer-related deaths worldwide [1]. In current clinical practice, patients are stratified for prognosis and treatment according to the tumor-node-metastasis (TNM) classification that is primarily based on histopathological features of the tumor [2]. In this TNM staging system, the ‘T’ describes the extension of the tumor through anatomical layers of the large intestinal wall, *i.e.* mucosa, submucosa, muscularis externa (m. propria) and serosa. The ‘N’ categorizes for the presence of tumor cells in the regional lymph nodes. The ‘M’ indicates whether distant metastasis is diagnosed, such as in distant lymph nodes, liver or lung. Overall, the prognosis of patients is inversely related with the stage of the disease (Table 1) [3,4]. Hence, early diagnosis has demonstrated to significantly decrease CRC mortality [5,6]. Recently, a nationwide screening program using a fecal immunochemical test (FIT) has been implemented in the Netherlands to facilitate early diagnosis and subsequent treatment. This test will be offered biennially to individuals in the age of 55 to 75. Following a positive test result, these participants will be referred for colonoscopy, to confirm presence of adenomas or cancer, and in case of the former also remove these [7]. Stage I-II CRC patients have a relative good prognosis (Table 1) and in general do not receive adjuvant chemotherapy after resection of the primary tumor according to the current guidelines from the American Society of Clinical Oncology [8]. Exceptions are stage II colon tumors with high-risk features, such as bowel obstruction or perforation, T4 tumors (penetration through the serosa), presence of vascular invasion, poorly differentiated tumors and less than 10 lymph nodes harvested and investigated [8]. In general, patients with stage III disease will receive systemic adjuvant chemotherapy. Also patients with stage IV disease qualify for systemic therapy, but frequently not with curative intent anymore. In addition to conventional systemic therapy (fluoropyrimidine combined with oxaliplatin or irinotecan), targeted therapy that specifically interferes with tumor-specific features, *i.e.* monoclonal antibodies and small

Table 1 | Stages and survival of CRC. Stages are according to the fifth edition of the TNM-system that is currently used in the Netherlands.

	T-stage	N-stage	M-stage	5-year cancer specific survival [4]
Stage I	T1-2	N0	M0	> 90%
Stage II	T3-4	N0	M0	App. 80%
Stage III	Any T	N1-2	M0	App. 60%
Stage IV	Any T	Any N	M1	< 10%

T1: tumor is confined to the submucosa; T2: tumor has not grown through the muscularis externa; T3: tumor has grown into the serosa; T4: tumor has penetrated the serosa and the peritoneal surface
 N0: no regional lymph nodes positive for tumor presence; N1: presence of tumor cells in up to three regional lymph nodes; N2: presence of tumor cells in more than three regional lymph nodes
 M0: no distant metastasis of the tumor; M1: the tumor has metastasized to distant organs

molecules, is administered to patients with unresectable metastatic CRC [3]. In current clinical practice, anti-EGFR (*e.g.* cetuximab or panitumumab), anti-VEGF (*e.g.* bevacizumab) and multi-kinase inhibitor (*e.g.* regorafenib) therapies have been implemented in the guidelines in the Netherlands. Anti-EGFR therapy is limited to *RAS* wild type tumors, because activating mutations in downstream targets of *EGFR* like *RAS* are strong predictors of poor response to anti-EGFR agents [3,9-11].

The widely used TNM staging system also has limitations. Since clinical outcome still varies between tumors that are classified within the same TNM stage according to the TNM system the main challenge is to achieve optimal prognosis prediction in stage II-III and therapy prediction in stage IV CRC [5]. Molecular biomarkers could facilitate better tumor stratification in order to ultimately improve patients outcome [12].

HISTOLOGY AND PATHOGENESIS OF COLORECTAL CANCER

The luminal side of the large intestine is covered with a single layer of epithelial cells, organized in the crypts of Lieberkühn. Within these crypts, the multipotent stem cells locating at the bottom compartment generate cells that rapidly divide, differentiate and migrate towards the lumen where the cells will exfoliate and undergo apoptosis [13]. This continuous self-renewal process of epithelial cells facilitates maintenance of the luminal surface of the large intestine [14,15]. Generally, tumor growth is the net result of the imbalance between cell proliferation and cell death. The vast majority of CRCs are adenocarcinomas, which originate from these epithelial cells [16,17]. Strong evidence supports that CRCs develop via a primary precursor lesion, which is called an adenoma. Only a fraction of the adenomas are assumed to ultimately transform into a malignant lesion. Tumor evolution is characterized by acquisition of specific biological capabilities disrupting critical physiological processes. Hanahan and Weinberg postulated six hallmarks of cancer comprising: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [18]. Avoiding immune destruction and deregulating cellular energetics are two emerging hallmarks [18]. Tumor-promoting inflammation, and genome instability are enabling mechanisms to accomplish these biological capabilities that characterize cancer [18].

GENOME INSTABILITY IN COLORECTAL CANCER

It has been established for decades that cancer is a genetic disease [19-21]. The colorectal adenoma-to-carcinoma progression, which has been firstly described by Muto *et al.* in 1975 [22], is characterized by the accumulation of somatic DNA aberrations [23]. In CRC, two molecular phenotypes of genome instability are distinguished at the DNA level. Approximately 15% of CRCs are microsatellite instable (MSI) and are characterized by mismatch repair (MMR) deficiency

generally through inactivation of *MLH1*, *MSH2*, *PMS2* or *MSH6* [24]. The majority of the microsatellite stable (MSS) tumors exhibit chromosomal instability (CIN), which results in gains and losses of relatively large chromosomal segments [25]. This may be attributed to deficient control of genome integrity by for instance *TP53* mutation, which could thus indirectly contribute to CIN [26]. Loss of *TP53* function dominates the landscape of cancer mutations [27-29] and is considered to be a rate-limiting step for malignant transformation of MSS CRC [23]. Recently, a subset of CRCs has been described with high mutation rates, *i.e.* more than 12 somatic mutations per one million bases [30]. The subgroup of MSS hyper-mutated CRCs are enriched for *POLE* aberrations, which may be the causal mutation for this phenotype in MSS CRC [30]. In addition, DNA promoter hyper-methylation is a common phenomenon in CRC, and a class of CRCs has been proposed based on methylation status, *i.e.* CpG Islands Methylator Phenotype (CIMP). Although it is established that hyper-methylation exists in a subset of tumors, it is still under debate whether this should be considered a distinct group of CRC. The CIMP and hyper-mutated tumors for instance largely overlap with the MSI phenotype [30].

MOLECULAR PATHOGENESIS OF COLORECTAL CANCER

Somatic DNA aberrations drive tumor formation and progression. Therefore, the variety in prognosis between cancer patients is likely to be reflected by heterogeneity across individual CRC genomes. Consequently, characterization of irreversible DNA aberrations may improve patient stratification for prognosis and therapy prediction [31]. Mutations in *APC* are the most common gene mutations in CRCs and are associated with early colorectal tumor development [15,23,28,30]. *APC* is a key negative regulator of WNT signaling that plays an essential role in control of proliferation and differentiation of intestinal epithelial cells [14,32]. The WNT signaling pathway is up-regulated in more than 90% of CRCs [30]. In MSS CRCs, mutations in *KRAS* and *TP53* are established to be involved in adenoma-to-carcinoma progression in addition to *APC* mutations [15,23]. While *APC* and *TP53* mutations did not evidently affect survival of CRC patients, tumors harboring *KRAS* activating mutations were associated with poor response to anti-EGFR therapy and has been inconsistently correlated with worse prognosis [33-35]. Additionally, somatic copy number aberrations (CNAs) that were associated with tumor adenoma-to-carcinoma progression are losses of chromosome 8p, 15q, 17p and 18q and gains of chromosome 8q, 13q and 20q affecting oncogene and tumor suppressor gene dosages [15,36-42]. One example of such a tumor driver is *AURKA* that is encoded at chromosome 20q, which is recurrently gained in more than 60% of MSS colorectal tumors [30,36,43,44]. *AURKA* overexpression promotes colorectal carcinogenesis and is moreover associated with poor prognosis in advanced CRC [36,38,41,45,46]. Colorectal MSS *versus* MSI tumors differ both in genotype as well as in clinic behavior. *TGFB2* frame-shift mutations are observed in about 90% of MSI tumors contributing to carcinogenesis by regulation of cell proliferation [15,24,47]. While *KRAS* is more frequently mutated than *BRAF* in MSS CRC, the opposite is the case in sporadic MSI tumors. Furthermore, common point mutation activating *BRAF* is associated with poor

prognosis in MSI CRC [15,48-50]. Overall, it has been shown that patients with non-metastasized MSI tumors have a favorable prognosis compared to non-metastasized MSS tumors, while the opposite is the case when tumors are metastasized to distant organs (stage IV disease) [50,51].

CHROMOSOMAL STRUCTURAL VARIANTS IN CRC

Besides small nucleotide variants (SNVs) and quantitative effects of CNAs, somatic structural variants (SVs) could also activate oncogenes and inactivate tumor suppressor genes. SVs represent deletions, insertions, inversions, and intra- and inter-chromosomal translocations [52]. Somatic SVs are caused by inadequate repair of DNA double strand breaks (DSBs) [52]. Oncogene-induced replication stress (e.g. through *KRAS* mutation) induces DSBs and in absence of genome integrity control, for instance by loss of *TP53*, genomic instable tumor cells are allowed to continuously proliferate [53]. DSBs can be induced by extrinsic factors (e.g. chemicals), chromosome segregation errors, centrosome amplification and dysfunctional telomeres [25,54]. Two major repair mechanisms of DSBs are homologous recombination (HR) and non-homologous end joining (NHEJ). Since HR is considered quite accurate, most chromosomal rearrangements likely result from non-allelic HR or NHEJ [55-58]. Alternatively, replication-based mechanisms could be involved such as break-induced repair or fork stalling and template switching [57-59]. Some CRC samples harbor clusters of complex (intra-) chromosomal rearrangements, a phenomenon which is termed 'chromothripsis' [60,61]. The hypothesis is that this severe chromosome shattering and subsequent inadequate DNA repair driven by NHEJ occurred in one single event [62].

SVs were already observed decades ago by karyotyping and low-resolution SKY imaging [25,42]. However, these techniques were insufficient for detection of chromosomal breaks at gene level. Whole genome deep sequencing allows identification of chromosomal rearrangements at base pair level [63]. Series of CRCs analyzed this way still are rather small. This way, several in-frame fusion genes have been reported including *VTI1A-TCF7L2*, *NAV2-TCF7L1*, *CAD-ALK*, *C2orf44-ALK* and the R-spondin fusions *PTPRK-RSPO3* and *EIF3E-RSPO2* [30,64-67]. The R-spondin fusions activate the Wnt signaling pathway and are mutually exclusive with *APC* mutations, indicating that these translocations cause gain-of-function protein alterations. Although in-frame fusion genes may have great impact, mutation frequencies are relatively low in CRC (less than 10%). Though, higher frequencies can be found in particular subtypes of colorectal tumors such as *PTPRK-RSPO3* fusions that were present in approximately 30% of traditional serrated adenomas [68]. Alternatively, SVs can also cause loss-of-function alterations. Little is known about SVs that may comprise loss-of-function mutations, such as *LACTB2-NCOA2* [69]. Another example of this subgroup of SVs is the deletion of the stop codon of the *EPCAM* gene that results in a transcriptional read-through that causes hyper-methylation and consequently silencing of the adjacent mismatch repair gene *MSH2* [70].

Overall, these examples from sequencing efforts show that genes affected by SV breakpoints are a biologically significant category of gene mutations in CRC in addition to the well-established somatic SNVs and CNAs [20,71,72]. Whereas SNVs and DNA copy number changes have been examined extensively, the effects on genes involved in SVs are poorly characterized in CRC. Consequently, the putative impact of SVs in colorectal tumor development and clinical behavior may well be underestimated and deserves further investigation.

AIM AND OUTLINE OF THIS THESIS

CRC is caused by genetic aberrations. Therefore, insight in underlying molecular biology and impact on clinical outcome could be gained by comprehensive genome-wide tumor profiling of somatic DNA aberrations. Apart from epigenetic changes, DNA mutations that can be distinguished are SNVs, CNAs and SVs. This thesis is focusing on genes that are recurrently affected by SV breakpoints and aimed to investigate their prevalence and clinical relevance by examining large series of CRCs.

Chapter two describes a user-friendly computational method to systematically identify genes that are recurrently affected by SV breakpoints from DNA CNA profiles. The R-package 'GeneBreak' has been made available from Bioconductor (<http://bioconductor.org>). The non-random locations of CNA-associated breakpoints in the cancer genome and their impact on carcinogenesis and patient outcome were investigated in **chapter three**. In this study, we used a large series of high-resolution array-comparative genomic hybridization (aCGH) profiles of advanced CRC samples (n=352) from CAIRO and CAIRO2 phase III clinical trials [73,74]. In **chapter four** we report comprehensive cancer genome profiling, including SNVs, CNAs and recurrent breakpoint genes, using a selected series of MSS stage II and III colon cancer samples (n=114). Recurrent breakpoint genes that could facilitate the adenoma-to-carcinoma progression are described in **chapter five**. For this analysis, we used high-resolution aCGH profiles from 118 colorectal adenoma [75] and 466 colorectal carcinomas. *MACROD2* appeared to be the most prominent recurrent breakpoint gene in CRC. **Chapter six** describes the evaluation of the prognostic value of *MACROD2* protein expression by immunohistochemistry on tissue microarrays containing core biopsies from a cohort of stage II and stage III colon cancers (n=386). Finally, in **chapter seven** we discuss the results from all studies included in this thesis.

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