CHAPTER 8

SUMMARY AND GENERAL DISCUSSION
Summary

Colorectal cancer (CRC) is a major healthcare problem with high incidence and mortality rates [1]. During CRC carcinogenesis multiple (epi)genetic alterations accumulate which change the behaviour of tightly regulated normal cells into cancerous cells [2]. The development of CRC is preceded by a well defined precursor lesion, the adenoma. Adenomas are quite common in the population, but do usually not progress to CRC [3]. Although multiple alterations are known that contribute to CRC development in general [4,5], it is yet largely unknown which alterations drive the transition of adenomas into CRCs. Chromosomal instability (characterised by gross chromosomal alterations, such as copy number gains and losses) contributes to the accumulation of genetic alterations [2]. Gain of 20q is frequent in CRC, but not in adenomas, and strongly associated with colorectal adenoma-to-carcinoma progression [6]. Thus far, it is unknown which genes on chromosome 20q drive this gain and how they stimulate carcinogenesis.

The aim of this thesis was therefore to identify processes involved in colorectal adenoma-to-carcinoma progression and to identify genes on the 20q amplicon that drive 20q associated colorectal adenoma-to-carcinoma progression.

In chapter 1, background information is provided on CRC, biological and metabolic processes that play a role in carcinogenesis in general, normal colon and CRC development and chromosome 20q gain in relation to CRC.

Chapter 2 describes an expression microarray study of 37 adenomas and 31 CRCs which was analysed using different strategies to unravel the biology of adenoma-to-carcinoma progression. By comparing mRNA expression of individual genes, 96 genes were identified that were significantly upregulated (including SPON2, RGS16 and AURKA) and 152 genes that were significantly downregulated (including FAM55D and PRKACB) in carcinomas compared to adenomas. Gene-sets associated with ageing (of which senescence is a cellular feature; upregulated), mitosis and mitosis-related processes such as chromosome binding and segregation (processes that contribute to the maintenance of chromosomal stability; all upregulated), fatty acid metabolism (downregulated), chromosome location 4q22 (downregulated), 20q11 (upregulated) and 20q13 (upregulated) were found to be significantly differentially expressed in carcinomas compared to adenomas. These results support the notion that adenomas and carcinomas are distinct lesions; adenomas appear to lack important characteristics which are necessary for malignant behaviour.

In chapter 3 a selection of gene-sets representing the basic Hanahan & Weinberg hallmarks of cancer, i.e. biological processes which are believed to be essential for cancer development and / or progression, were compared between colorectal adenomas and carcinomas, using Gene Set Enrichment Analysis in the same data set as investigated in chapter 2. Several cancer-related biological processes were identified that appeared to be affected during colorectal adenoma-to-carcinoma progression and key genes within these pathways were identified. Gene-sets representing chromosomal instability, proliferation, differentiation, invasion, stroma activation and angiogenesis were found to be differentially expressed in CRCs compared to adenomas. No differences in expression were detected for the apoptosis, cell-cycle, hypoxia, tumour-associated macrophages, immune response and metastasis gene-sets. Moreover, in this study 18 key genes within
the differentially expressed gene-sets were identified which showed significantly increased expression levels (e.g. AURKA and PDGFRB; for which elevated expression was confirmed at the protein level). These genes may serve as tumour markers to improve molecular characterisation (e.g. identification of high-risk adenomas) of colorectal tumours. The identification of differentially expressed processes and genes has contributed to a better understanding of the biology of adenoma-to-carcinoma progression and demonstrates that adenomas and carcinomas are distinct lesions in terms of the activity of biological processes and molecular alterations.

Chromosomal instability is one of the processes that had significantly increased activity in CRCs compared to adenomas. Chromosomal instable tumours show gain and loss of chromosomal regions. Gain of chromosomal region 20q is frequent in CRC and usually involves a large part of the chromosome arm, suggesting that multiple genes on the 20q amplicon may contribute to 20q gain associated colorectal adenoma-to-carcinoma progression. Genes that show functional effects and which mRNA and protein expression correlate with chromosome 20q gain are believed to promote 20q gain associated colorectal adenoma-to-carcinoma progression. Therefore, in chapter 4 the effect of candidate genes located on 20q that may drive 20q gain associated colorectal adenoma-to-carcinoma progression on cancer-related processes (e.g. cell viability, anchorage-independent growth and invasion) was studied. Interference with multiple genes affected cell viability (e.g. AURKA, TPX2, SLC17A9, RBM39, TCFL5, CSE1L and PRPF6) and anchorage-independent growth (e.g. AURKA, TPX2, HM13, CSE1L and DIDO1). AURKA and TPX2 were also found to affect invasion. mRNA and protein expression of AURKA and TPX2 correlated with 20q DNA copy number status. Both AURKA and TPX2 were therefore classified as important genes in promoting 20q gain associated colorectal adenoma-to-carcinoma progression. These genes are located on different regions of chromosome 20q, which supports the idea that gain of the 20q region results in the activation of multiple genes that stimulate colorectal adenoma-to-carcinoma progression.

In chapter 5 the role of BCL2L1 in CRC carcinogenesis was further investigated. While carcinogenic features of this gene have been described in literature, BCL2L1 was not considered a candidate gene for 20q gain associated colorectal adenoma-to-carcinoma progression, since its DNA copy number status did not correlate with BCL2L1 mRNA expression. Yet, results from chapter 4 suggested that the carcinogenic effect of BCL2L1 could go beyond its role in apoptosis. Indeed, BCL2L1 was found to be functionally involved in several cancer-related processes; downmodulation of BCL2L1 was found to inhibit cell viability and anchorage-independent growth, but invasion was unaffected. BCL2L1 DNA copy number and protein expression were increased in CRCs compared to adenomas, but differences in BCL2L1 protein expression were even more pronounced when comparing tumours (adenomas and carcinomas) with and without 20q gain independent of phenotype. There was, however, no difference in mRNA expression between adenomas and carcinomas and no correlation of the copy number of the actual BCL2L1 locus with protein expression. The observed 20q-related BCL2L1 protein expression could therefore be regulated at the post-transcriptional level by a distinct factor on 20q (e.g. ZNF217, AURKA or miRNAs). Consequently, BCL2L1 itself is not likely a driver of chromosome 20q gain associated adenoma-to-carcinoma progression.

Of the other genes that showed functional effects on cancer-related processes antibodies were available for CSE1L, DIDO1 and RBM39. To reveal whether these genes could drive 20q gain associated colorectal adenoma-to-carcinoma progression, in chapter 6
the differences in mRNA and protein levels between colorectal adenomas and carcinomas were analysed in relation to 20q. Based on protein expression, which was increased in CRCs compared to adenomas but not in tumours with 20q gain, CSE1L does not seem to be a driver of chromosome 20q gain. mRNA expression levels of DIDO1 and RBM39 indicate that the expression of these genes is associated with 20q gain. Unfortunately, this could not be confirmed at the protein level because most tumours showed strong protein expression overall. Understanding the biology of colorectal adenoma-to-carcinoma progression is important to improve clinical practice. A secure option to reduce CRC incidence and mortality is to detect and remove all adenomas. This would, however, result in a major overtreatment of patients. In chapter 7 the relevance of the identification of the subgroup of colorectal adenomas (about 5% of all adenomas) that have a high risk to turn malignant is described. Classical morphological characteristics fail to accurately discriminate between adenomas that will become malignant and those that will not. Knowledge of biological and metabolic processes that are changed between adenomas and carcinomas and the genes involved is believed to be beneficial in adenoma stratification. This information will help to develop triage tests that identify individuals at high probability of having truly high-risk colorectal adenomas. These tests could be used in a screening setting whereby colonoscopy is reserved for those patients that bear high-risk adenomas.

**Colorectal cancer development: a refined model**

Based on current literature and results from recent experiments and analyses which are described in this thesis and are summarised above, we present a refined model of colorectal cancer development and progression (Figure 1). This model describes cancer-related and metabolic processes that contribute to different stages of CRC development and genetic alterations that are frequently involved.

**Future perspectives**

Despite our improved understanding of CRC carcinogenesis, the model of colorectal adenoma-to-carcinoma development is still far from complete. Major progress has been made concerning genes on chromosome 20q that contribute to CRC progression. In this thesis the effect of downmodulation of 20q genes by siRNAs has been investigated on several cancer processes, e.g. cell viability, anchorage-independent growth and invasion. It would also be interesting to analyse the effect of these candidate genes on other cancer processes involved in CRC progression, such as those that have been identified by pathway analysis as being differentially expressed between adenomas and CRCs (e.g. senescence, proliferation, apoptosis, differentiation, chromosomal instability and angiogenesis). In addition, the role of these genes in the development of CRC metastasis could be investigated. Furthermore, the results obtained need further confirmation before the conclusion can be made that these genes really drive colorectal adenoma-to-carcinoma progression. To really prove this point, overexpression of the gene(s) in benign adenoma cells should cause malignant transformation. This is an easy statement to make, but very difficult to investigate in practice. Adenoma cells are extremely difficult to culture and only sporadic reports exist in literature that claim to be successful [7-9].

Since 20q gain generally involves the whole chromosome arm, the hypothesis is that
CRC development and progression model

**Normal mucosa**

- Activation Wnt pathway → adenoma formation
- Additional mutations → adenoma outgrowth

**Adenoma**

- Senescence: TP53
- Proliferation / viability: PLK1, CCNF, AURKA, TPX2, SLC17A9, RBM39, TCF6, C3E1L, PRPF6, BCL2L1, miR-17-92, miR-21, miR-143, miR-145
- Differentiation: ADIR1, NUDT1
- Chromosomal instability: AURK1, G20orf24, TPX2
- Invasion: SPARC, DCN, PDGFRB, AURKA, TPX2, miR-21
- Stroma activation: SSCCA1, ID3, LUM

**Carcinoma**

- Fatty acid metabolism (β-oxidation): ADH1C, HADHB, ACADS

**Figure 1** Colorectal cancer development and progression model. This model describes cancer-related and metabolic processes that contribute to adenoma development from normal colon mucosa and to adenoma-to-carcinoma progression. Genetic alterations that are involved in aberrant activity of these processes are indicated. Genes that are located on chromosome arm 20q are underlined.

CRC development from normal colon epithelium is initiated by disruption of normal tissue homeostasis, a process in which the Wnt, BMP/TGF-β and Notch signalling pathways play an important role. The balance of proliferation, differentiation and apoptosis is disturbed through constitutive activation of the Wnt signalling pathway. Wnt signalling is activated mainly by inactivation of the APC tumour suppressor gene and in some cases by activation of β-catenin. Overexpression of miR-135, which suppresses APC expression, may also contribute to activated Wnt signalling. The resulting small adenoma requires additional genetic changes to enlarge; e.g. mutations in KRAS stimulate the outgrowth of adenomas. The growing mass of cells becomes hypoxic. Hypoxia as well as the high proliferation rate of adenoma cells shifts metabolism in favour of glycolysis. In addition, oxidative phosphorylation is reduced and pyruvate metabolism enhanced (e.g. as demonstrated by increased LDHA expression). These metabolic alterations guarantee the production of the energy and macromolecules which are needed for rapid proliferating cells. At the genomic level alterations are already building up in these adenoma cells; mutations can be detected and copy number alterations are observed at a low level. These continuously proliferating cells are limited by senescence, a process which drives cells in a state of irreversible growth arrest. Overcoming senescence requires stabilisation of telomeres - mainly by activation of hTERT -, a defect DNA damage response and alterations in signalling pathways. The minority of cells that succeed in crossing the senescence barrier of growth arrest can accumulate additional genetic alterations which are needed to further stimulate carcinogenesis, e.g. progression of colorectal adenomas into carcinomas. Inactivation of p53 might help to overcome senescence and seems to play a role in malignant transformation. The majority of the adenomas, however, do not progress into cancer; malignant transformation only takes place in about 5%. Genomic instability allows the accumulation of genetic alterations and therefore plays an important role in malignant transformation of colorectal tumours. Two major pathways of genomic instability contribute to adenoma-to-carcinoma progression: microsatellite instability (15% of cases) and chromosomal instability (85% of cases). Chromosomal unstable
multiple genes on 20q contribute to colorectal adenoma-to-carcinoma progression. In this thesis it is shown that multiple 20q genes influence cancer processes in a 20q dependent manner. To what extent these genes individually affect cancer processes, or whether they have a synergistic effect, meaning that two or more genes need to be amplified in order to obtain the carcinogenic phenotype, remains to be resolved. By downregulating 20q genes it is expected that genes that affect cancer processes either individually or synergistically can be identified. Overexpression studies (e.g. by Microcell Mediated Chromosome Transfer - MMCT -, human chromosome fragments or human artificial chromosomes - HACs -) could further demonstrate synergy between candidate driver genes on 20q in CRC carcinogenesis. Furthermore, new techniques might help to identify other relevant genes (including miRNAs and gene transcripts) that may contribute to colorectal adenoma-to-carcinoma progression. Alternative splicing and alternative polyadenylation are important regulatory mechanisms of gene expression [10-14]. Therefore, genome-wide analyses of alternative splicing and alternative polyadenylation may provide important information on those specific gene transcripts that may contribute to (20q gain associated) colorectal adenoma-to-carcinoma progression. Massively parallel sequencing (MPS) can be used to identify genetic alterations such as mutations, copy number alterations and miRNA profiles [15-17]. Identification of gene mutations by MPS may give clues about other candidate genes on chromosome 20q that may contribute to colorectal adenoma-to-carcinoma progression. In addition, expression of miRNAs can be affected by gain of the 20q region and may affect colorectal adenoma-to-carcinoma progression. This strategy can also be used to identify candidate genes (including miRNAs) for other relevant chromosomal regions that are frequently gained in colorectal carcinogenesis such as 8q and 13q.

Unravelling the biology of colorectal adenoma-to-carcinoma progression is very relevant from a clinical point of view. Understanding the biology of CRC carcinogenesis may contribute to the development of a specific molecular screening test and the identification of therapeutic targets. To detect CRC in an early stage, population screening for CRC...
using FIT (Fecal Immunochemical Test) will start in the Netherlands in 2013. The FIT can detect hemoglobin, a blood protein that is released in the stool e.g. by bleeding colorectal tumours [18]. Individuals with a positive test are referred for colonoscopy. Unfortunately, FIT is suboptimal in terms of specificity and sensitivity. Genes identified in this thesis as playing a role in colorectal adenoma-to-carcinoma progression could be of value in a molecular screening test for CRC and high-risk adenomas with an excellent positive and negative predictive value. Such a test will help to reserve colonoscopy to diagnose individuals with CRC and to remove high-risk adenomas, thereby preventing major overtreatment.

In addition, genes that show carcinogenic effects when amplified are of interest as therapeutic targets. AURKA, identified in this thesis as one of the main driver genes of chromosome 20q gain, is under evaluation as therapeutic target [19,20]. Recently, TPX2, next to AURKA in this thesis as the other major driver gene of chromosome 20q gain, has also gained attention as therapeutic target [21]. Inhibition of AURKA and TPX2 gene expression may have additional effect when combined with other drugs [21,22]. The apoptosis regulating gene BCL2L1 that has been found to contribute to several cancer-related processes and which protein expression seems to be regulated in a 20q dependent manner is another interesting drug target. Overexpression of the anti-apoptotic Bcl-x isoform of BCL2L1 is an important cause for chemotherapy resistance [23-26] and could be reduced by inhibition of Bcl-x. Inhibitors of Bcl-x by themselves have pro-apoptotic and anti-carcinogenic effects [27-29]. In addition, they can be combined with other chemotherapeutic drugs [30,31].

In conclusion, the results obtained in this thesis have contributed to an improved understanding of the molecular biology of colorectal adenoma-to-carcinoma progression. This knowledge has the potential to improve clinical practice at multiple levels.

References