Summarizing discussion and future perspectives
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In the Netherlands, colorectal cancer (CRC) incidence ranks third in men and second in women and it is the second most common cause of death due to cancer. Colorectal carcinogenesis is driven by an accumulation of genetic and epigenetic events, which affect gene expression, eventually leading to changed protein expression or aberrant function of proteins that ultimately result in uncontrolled cell growth and behavior. Chromosomal instability, i.e. numerical or structural chromosomal changes, is the most common type (~85 percent) of genetic abnormality observed in sporadic colorectal cancer.

The aims of this thesis were to investigate chromosomal alterations in colorectal cancer and its precursor lesions, i.e. adenomas, and to explore the potential of DNA copy number changes to address the main clinical needs in colorectal cancer at the levels of screening, prognosis assessment in primary CRC and predicting response to therapy in advanced CRC. In all chapters of this thesis comparative genomic hybridization (CGH) technique was used. CGH enables detection of genome-wide chromosomal copy number changes in a single experiment, without the need for cell culturing. Another advantage of this technique is that DNA obtained from formalin-fixed paraffin-embedded (FFPE) tissue archives can be applied. In chapter 2 to 5 chromosome-based CGH was used detecting rather large genomic regions of approximately 10 Mb. In chapter 6 to 8 array-based CGH was used and because of its higher resolution (0.1 Mb to 1 Mb) and sensitivity in comparison to conventional CGH, it allowed us to narrow down chromosomal regions of interest and detect copy number changes of specific genes.

Chapter 2 presents a large-scale study on chromosomal alterations in 194 colorectal tumor samples of various stages of cancer development analyzed by chromosome-based CGH and focuses on those alterations that are associated with adenoma to carcinoma progression. As only 5 percent of colorectal adenomas will ever progress to cancer, the first aim was to find genetic changes capable of discriminating adenomas with and without a high risk of progression, and secondly to refine the current genetic models of colorectal adenoma to carcinoma progression. The overall number of chromosomal abnormalities correlated to dysplasia, but not to mutations in APC or KRAS. Accumulation of losses at 8p21-pter, 15q11-q21, 17p12-p13 and 18q12-q21, and gain at 8q23-qter, 13q14-q31 and 20q13, was strongly associated with adenoma to carcinoma progression, independent of degree of dysplasia. These DNA copy number changes could therefore possibly serve as markers of a high progression risk in adenomas. Unsupervised hierarchical cluster analysis demonstrated in the adenomas the presence of three distinct, frequently occurring combinations
of genetic alterations: 17p loss and KRAS mutations, 8q and 13q gain, and 18q loss and 20q gain, respectively. This suggests that specific combinations of a few abnormalities, rather than a mere accumulation of events are important for tumor progression. These observations are consistent with the existence of multiple independent chromosomal instability pathways of colorectal cancer progression, each of which may possibly disrupt similar biological processes, such as proliferation, differentiation, apoptosis, and invasion, in different manners.

Initiation and progression of cancer also involves next to genetic alterations, epigenetic alterations such as DNA methylation, and these genetic and epigenetic alterations interact in driving the development of cancer [1]. However, the precise timing and interrelationship of these changes in colorectal carcinogenesis have not yet been fully elucidated [2]. Chapter 3 describes a study investigating the timing of promoter methylation and its relationship with mutations and chromosomal alterations in colorectal normal mucosa, adenomas and carcinomas. Promoter methylation status of 9 tumor suppressor and DNA repair genes associated with colorectal cancer development was evaluated in the same dataset as used in chapter 2 using methylation-specific PCR (MSP). This revealed that a high frequency of promoter methylation for the majority of these genes is already present in adenomas without any histological signs of progression and that this level of methylation remained stable with tumor progression. Even in morphologically normal mucosa from colorectal cancer patients, promoter methylation of the majority of the genes studied was observed, but in lower frequencies compared to the carcinomas. We concluded that promoter methylation can be regarded as an early event in colorectal carcinogenesis, preceding chromosomal alterations. It is tempting to speculate that promoter methylation of cell cycle control genes would actually be associated with the initiation of chromosomal instability. However, for the relevant genes studied in the present study, i.e. cell cycle regulator p16\textsuperscript{INK4A} and the mitotic checkpoint control gene CHFR, promoter methylation status showed no association with chromosomal instability.

Ever since the polyp-cancer sequence was postulated, the existence of an alternative route to colorectal cancer has been proposed. In the last decade it has become clear that flat adenomas represent an alternative phenotype of colorectal cancer precursors, next to polypoid adenomas [3-5]. Compared with polypoid adenomas of similar size, flat adenomas show a higher frequency of high-grade dysplasia [6,7] and rapid submucosal invasion. Based on the concept that a tumor’s phenotype is driven by its genotype, we wanted to explore whether the patterns of chromosomal alterations in flat adenomas would differ from those observed in polypoid adenomas. In Chapter 4 a small series of flat adenomas and carcinomas were analyzed by chromosome-based CGH and their copy number profiles were compared to polypoid tumors analyzed in chapter 2. Flat tumors were marked by a high prevalence of 18q loss and 20q gain. This correlation between 18q loss and 20q gain had been observed in a subgroup of polypoid colorectal tumors described in
chapter 2, and the presence of these chromosomal alterations correlated with higher grades of dysplasia and a higher risk of progression to colorectal cancer [8]. In addition, multiplex ligation-dependent probe amplification (MLPA) for identifying DNA copy number changes of multiple individual genes on chromosome 20 was performed on flat and polypoid tumors. Both flat and polypoid colorectal tumors with 20q gain showed similar patterns of copy number ratios for the individual genes tested. We concluded that flat colorectal tumors resemble one of the distinct patterns of chromosomal changes found in polypoid colorectal tumors, and that this pattern could convey a more aggressive clinical behavior.

Adjuvant chemotherapy for colon cancer patients with unfavorable characteristics, i.e. presence of lymph node metastases (TNM stage III) has proven to be beneficial. Adjuvant chemotherapy for stage II colon cancer remains controversial [9-11]. Patients with stage II tumors constitute a particularly heterogeneous population that harbors a subgroup of patients that do develop metastatic disease and could benefit from adjuvant chemotherapy. In chapter 5 we investigated which chromosomal alterations are related with patient survival in a small series of colorectal carcinomas studied by chromosome-based CGH. In this series, carcinomas with gain of 20q had a significantly worse survival than tumors without 20q gain. We concluded that gain of 20q could be an indicator of poor clinical outcome in colorectal cancer patients which could have clinical implications especially in patients with stage II cancer.

As summarized above, gain of 20q was found to be one of the most frequent chromosomal alterations in colorectal adenoma to carcinoma progression and in adenomas with a more aggressive clinical behavior, and in carcinomas this alteration was related to worse patient survival. In order to try to identify the pivotal oncogenes located at chromosome 20q, in chapter 6 the gene dosage effect was studied in a series of 149 colorectal tumor samples using array-based CGH and expression microarray analysis. Chromosome 20q was frequently altered in the progressed adenomas and carcinomas (in 60% of cases) and in the nonprogressed adenomas, gains of 20q were only detected in 20% of cases, supporting a role for 20q gain in colorectal adenoma to carcinoma progression consistent with earlier observations (chapter 2). Narrowing down the gained region by array-based CGH yielded three SROs: one at 20q11.22-q11.23, and the other two at 20q13.32-q13.33 and 20q13.33. A robust analysis of altered expression of genes identified 7 genes C20orf24, AURKA, RNPC1, TH1L, ADRM1, C20orf20 and TCFL5, mapping at 20q significantly overexpressed in carcinomas compared to adenomas as consequence of copy number gain of 20q. Increased mRNA expression of these genes was confirmed by qRT-PCR and increased protein expression for AURKA (aurora kinase A) by immunohistochemistry. These genes may therefore be important in chromosomal instability-related adenoma to carcinoma progression and may serve as
prognostic markers for colorectal cancer. AURKA is especially of interest because it is involved in cell cycle regulation and its overexpression induces centrosome amplification and aneuploidy [12].

Approximately 50 percent of all colorectal cancer patients develop distant metastases and will ultimately die of this disease. While new drug regimens have resulted in increased response rates and prolonged median survival, treatment strategies are still mainly based on a 'one size fits all' approach to which only a subset of patients will respond. Therefore, predictive markers are needed to identify those patients who will maximally benefit from the available treatment options. Colorectal cancer biologically is a heterogeneous disease and this heterogeneity may affect response to drug therapy. Chapter 7 presents an array-based CGH analysis of 32 primary tumors of patients with advanced colorectal cancer. Correlation of genome wide DNA copy number profiles of primary tumors with response to first-line combination chemotherapy with capecitabine and irinotecan was investigated. Unsupervised hierarchical cluster analysis of the tumors yielded two clusters, and cluster membership showed a significant correlation with response status. Nonresponders had fewer chromosomal alterations compared to responders, in particular fewer losses were found. Most prominent differences between the two groups were losses of regions 18p11.32-q11.2 and 18q12.1-q23, which were more frequently observed in responders. These findings indicate that advanced colorectal cancer patients are a heterogeneous group at the level of genome wide DNA copy number status, and that these differences are relevant for the response to chemotherapy.

For another type of drug therapy, i.e. EGFR inhibitors like cetuximab and panitumumab, it has been shown recently that presence of \textit{KRAS} mutations in tumor tissue predicts poor response [13-16]. However, also response rates in colorectal cancer patients with wild-type \textit{KRAS} are still low, indicating that other factors, next to \textit{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 \textit{KRAS} mutation occur and if we could determine if within the category of wild-type \textit{KRAS} carcinomas, heterogeneity exists at the level of DNA copy number changes. To this end, array-based CGH data of sixty-four primary colorectal cancers and expression microarray data of thirty-four colorectal tumors were analyzed for association with \textit{KRAS} mutation status and gene dosage effects of relevant genes were evaluated. Candidate markers were validated on an independent series of 35 colorectal carcinomas. The most significant difference between colorectal cancer patients with or without mutated \textit{KRAS} was chromosomal region 8q23.3-q24.3, where \textit{MYC} and \textit{FAK} co-localize. DNA copy number gains of \textit{MYC} and \textit{FAK} were significantly more frequent in the wild-type \textit{KRAS} tumors. Of these two genes only \textit{FAK}, which is an upstream regulator of the RAS signaling pathway [17,18], showed a positive correlation between copy number gain and mRNA
overexpression in both learning and validation set. This could indicate that continuously activated RAS signaling in CRC can not only be induced by mutated KRAS, but also by FAK overexpression as a result of a gene dosage effect. This then leads to the hypothesis that CRC patients whose wild-type KRAS tumor contains FAK copy number gain may fail to benefit from anti-EGFR therapy, like CRC patients whose tumors carry a KRAS mutation.

Future perspectives

In this thesis we described 7 cancer-associated DNA copy number changes that turned out to be strong indicators of progression towards malignancy in colorectal adenomas and may therefore be used as markers of high risk adenomas. The ability to distinguish these high risk adenomas (~5% of the adenomas) could be highly relevant for colorectal cancer screening. Especially gain of chromosome 20q is of interest, because it is not only one of the most frequent chromosomal alterations in colorectal adenoma to carcinoma progression but was also associated with the more aggressive subgroup of polypoid and flat adenomas, and CRC patients with gain of 20q also had a worse survival. Thus genes at 20q, or their proteins, could serve as markers for early detection of colorectal cancer in stool or blood. Alternatively, they may serve as new possible targets for pharmaceutical intervention in the development of colorectal cancer. In chapter 6 we found 7 genes (C20orf24, AURKA, RNPC1, TH1L, ADRM1, C20orf20 and TCFL5) located on 20q to be overexpressed due to gene dosage effects. For AURKA, overexpression at the protein level was confirmed by immunohistochemistry. Unfortunately at the time of the study, for the proteins of the other 6 genes no immunohistochemical antibodies were or not available or they were not optimized yet for immunohistochemical staining. However, in the near future also for the majority of these genes immunohistochemistry experiments are planned, because at the moment we are developing some antibodies against these proteins. Currently, functional studies are ongoing to discover the biological relevance of these 7 genes.

Colorectal cancer biologically is a heterogeneous disease at the DNA, RNA and protein level which may affect response to drug therapy. Our data and those of others [19-23] show that DNA copy number profiles can predict response to therapy. In chapter 7 we described that CRC patients with loss of chromosome 18 respond better to combined capecetabine and irinotecan therapy. However, this chromosome harbors many genes and which of these are the candidate genes responsible for this response phenotype is unknown. To learn whether these findings can be of actual value in the selection of patients for chemotherapy, a large-scale validation study is necessary, which is presently ongoing. In this study, a higher-resolution array CGH platform is being
used that will allow to identify in more detail which individual genes are associated with response to drug therapy. In chapter 8 we found that copy number gain of FAK is a potential marker of response to EGFR-targeted therapy in wild-type KRAS colorectal cancer. Also here a large-scale validation study of CRC patients treated with cetuximab or panitumumab with follow-up data will be necessary to prove whether copy number gain of FAK actually will turn out to be a predictive marker for selecting patients for EGFR-targeted therapy.

While histopathology will maintain as a cornerstone of cancer diagnosis, biological features of tumors will inevitably become important as diagnostic, prognostic and predictive markers. However, array-based CGH will probably be no longer the method of choice for high throughput DNA copy number profiling, because of the arrival of massively parallel sequencing (MPS), which is the latest technology to screen genomic instability. This technique offers the possibility of genome-wide screening for point mutations, DNA copy number changes and rearrangements in one single experiment [24]. In the near future, we will be able to read the complexity of colorectal cancer biology and use this acquired biologic information to develop cost-effective tests for better diagnosis of CRC patients and tailoring therapy.

References