

Chromosomal instability in flat adenomas and carcinomas of the colon

Journal of Pathology 2005, 205: 514-521

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CHAPTER 4

Abstract

Background: Flat adenomas are flat or slightly elevated dysplastic lesions of the colorectal mucosa, mostly with a tubular architecture. Compared with polypoid adenomas of similar size, flat adenomas show a higher frequency of high-grade dysplasia and rapid submucosal invasion. The aim of this study was to survey whether flat colorectal lesions differ in their pattern of chromosomal aberrations from their polypoid counterparts.

Methods: Six flat adenomas and 12 flat carcinomas were analysed by comparative genomic hybridisation (CGH) and the pattern of chromosomal aberrations was compared to a previously published series of 112 polypoid adenomas and 82 polypoid carcinomas. In addition, multiplex ligation-dependent probe amplification (MLPA) for identifying DNA copy number changes of 25 individual genes on chromosome 20 was performed on 14 flat and 15 polypoid tumours.

Results: With CGH, flat adenomas showed on average 1.8 gains (range 1-4) and 3.2 losses (range 0-4), and the flat carcinomas 4.5 gains (range 0-8) and 3.5 losses (range 1-6). In both adenomas and carcinomas a high frequency of 20q gain (83% and 92%, respectively) and 18q loss (83% and 92%, respectively) were found. This correlation between 20q gain and 18q loss had previously been observed in a subgroup of polypoid colorectal tumours. Both flat and polypoid colorectal tumours with 20q gains by CGH showed similar patterns of copy number ratios for the individual genes tested. *TOPI*, *BCL2L1* and *E2F1* had median copy number ratios of 2 or higher, while *ZNF217* had a ratio around 3.

Conclusion: Flat adenomas and carcinomas of the large intestine show a similar pattern of chromosomal aberrations as a specific subgroup of polypoid lesions. The transcription factor *ZNF217* is an important candidate for driving the 20q gain.

Introduction

Adenocarcinomas of the large intestine arise through a dysplastic precursor stage called adenomas. For at least two decades, the term adenoma and polyp have been used as synonyms and it was thought that all colorectal cancers, unlike cancers of e.g. oesophagus and stomach, would arise from polypoid lesions [1]. However, starting in Japan, general awareness has grown that adenomas can also present as flat dysplastic lesions, called flat adenomas [2-4]. Endoscopically flat adenomas are flat or slightly elevated lesions, sometimes with a central depression, and often 'reddish' in colour. Histologically, most flat adenomas have a tubular architecture. Compared with polypoid adenomas of similar size, flat adenomas show more frequently high-grade dysplasia [3,5]. Furthermore, these lesions show rapid submucosal invasion.

The paradigm of genetic changes in colorectal adenoma to carcinoma progression is largely based on findings in polypoid adenomas [6,7]. In addition to the Vogelstein model, several other genetic changes have been demonstrated to play an important role in colorectal adenoma to carcinoma progression, especially occurring at the chromosomal level [8-10]. In a large series of colorectal adenomas and carcinomas analysed by comparative genomic hybridisation (CGH), seven chromosomal aberrations (loss at 8p21-pter, 15q11-q21, 17p12-13 and 18q12-21, and gain at 8q23-qter, 13q14-31 and 20q13) appeared to be specifically associated with adenoma to carcinoma progression. In addition, hierarchical cluster analysis of all adenomas demonstrated the presence of three distinct subgroups of adenomas, marked by characteristic combinations of genetic aberrations in the adenomas; one cluster was marked by 17p12-13 loss and *KRAS* mutation, one by 8q23-qter and 13q14-31 gain, and one by 18q12-21 loss and 20q13 gain. Clustering of all carcinomas yielded two subgroups. One group showed among other abnormalities, a high frequency of 17p12-13 losses and a low frequency of 18q12-21 loss and 20q13 gain, while the other group showed a reverse pattern.

Based on the concept that a tumour's phenotype is driven by its genotype, the present study aimed to test the hypothesis that flat colorectal adenomas and carcinomas show different patterns of chromosomal aberrations compared to their polypoid counterparts. To this end, DNA copy number changes were studied in a series of flat adenomas with high-grade dysplasia as well as early flat carcinomas by means of comparative genomic hybridisation (CGH) and multiplex ligation-dependent probe amplification (MLPA), and compared to the results found in polypoid colorectal adenomas and carcinomas [10].

Material en Methods

Material

Formaldehyde-fixed paraffin embedded tissue samples from 6 flat adenomas and 12 flat early (pT1) carcinomas of the colon were obtained from the archives of the Institute of Pathology, Klinikum Bayreuth, Bayreuth, Germany. Of the six adenomas, five were of the tubular type, and one showed a tubulovillous architecture. All adenomas showed severe dysplasia. In one case, next to the adenoma a very small focus of moderately differentiated carcinoma was present. On endoscopy all these lesions impressed as flat, and they matched the histological criteria for flat colorectal neoplasms [3,5]. Of the 12 carcinomas 4 were well differentiated and 8 were moderately differentiated. In addition, from a large series of colorectal adenomas and carcinomas previously studied by comparative genomic hybridisation, fifteen polypoid colorectal tumours (2 adenomas and 13 carcinomas) with 20q gains were selected for MLPA. These lesions on endoscopy had a polypoid or exophytic appearance. Archival material was used in compliance with the institutions ethical regulations.

Comparative Genomic Hybridisation.

DNA isolation and comparative genomic hybridisation were performed as described before [11]. In short, tumour and reference DNA were labelled by nick translation with biotin-16-dUTP and digoxigenin-11-dUTP (Boehringer, Mannheim, Germany), respectively, and hybridised to normal human metaphase chromosomes. Haptens were stained with avidin-FITC and anti-digoxigenin-TRITC and image acquisition and analysis was performed with the Cytovision CGH software package 3.5 (Applied Imaging, Sunderland, England). Averaged fluorescence ratios and their 95% confidence intervals were plotted along ideograms of the corresponding chromosomes in a so-called relative copy number karyotype. Chromosomal gains or losses were interpreted when the fluorescence ratio was significantly higher or lower than 1.0, as evaluated by the 95% confidence interval. Excluded from consideration were chromosome region 1p32-pter, and chromosomes 16, 19 and 22, because of their high content of repetitive sequences, which can affect the reliability of CGH results at these chromosomes.

Multiplex ligation-dependent probe amplification

MLPA is a quantitative multiplex PCR based approach that allows in a single experiment to determine the relative DNA copy number of up to 40 different targets at the resolution of individual genes, requiring only minimal amounts of DNA [12]. Details on probe sequences can be found on <http://www.mlpa.com>.

In MLPA, each probe is made up of two hemiprobcs consisting of a synthetic and a M13 derived oligonucleotide which contain target-specific sequences. The hemiprobcs are designed to hybridise immediately next to each other. Only after hybridisation they can be ligated to form one strand, which can be amplified by PCR. The ligated probes for the different genes 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 all probes in a set, PCR products can be sorted by capillary electrophoresis, allowing the measurement of relative DNA copy number of the individual target genes.

For the present study a dedicated oligonucleotide MLPA probe set for 25 genes on chromosome 20 was designed. Twenty of the 25 probes covered three different areas on the long arm of chromosome 20, i.e. 20q11, 20q13.1-q13.2 and 20q13.3, and the other 5 probes were located on the short arm of chromosome 20. The probe mixture further included 9 reference probes for normalization purposes (three probes on chromosome 2, three probes on chromosome 3 and one probe on chromosome 1, 5 and 12 each). Since in four cases after the CGH experiments no DNA was left anymore, MLPA could only be performed on 14 flat tumours (5 flat adenomas and 9 flat carcinomas).

Of every tumour 20-50 ng of DNA was denatured at 98°C for 5 min. MLPA hemiprobcs were added and were allowed to hybridise for 16 h at 60°C in a thermocycler. Then IU Ligase-65 enzyme was added and ligation was allowed to proceed for 10-15 min at 54°C. After heat inactivation of the ligase enzyme at 98°C, primers, dNTPs and Taq polymerase (Promega) were added and PCR amplification was performed for 33 cycles (30s at 95°C, 30s at 60°C and 1min at 72°C). Amplification products were detected and quantified by capillary electrophoresis using the ABI 3100 (Avant) capillary sequencer, with a ROX-labelled internal size standard (ROX-500 Genescan; Applied Biosystems, Warrington, United Kingdom).

MLPA data analysis

For each tumour, peak areas of every probe were determined for further analysis. For every probe, the median peak area obtained from at least three different PCR reactions was calculated. The reference median peak areas were obtained from normal formaldehyde fixed, paraffin embedded tissue samples from three different individuals, each of which were analysed at least three times independently. In every tumour, for every probe, a tumour to normal DNA copy number ratio was obtained by dividing the median area under the peak for each probe in the tumour tissue by the median value of the same peak for the reference DNA. Next all ratios were normalized by

setting the median tumour to normal DNA copy number ratio of the reference genes in the probe mixture to 1.0. A ratio lower than 0.7 was considered a deletion and a ratio higher than 1.3 a gain. Descriptive statistics of numbers of gains and losses were obtained, and represented as a heatmap (<http://quertermous.stanford.edu/heatmap.htm>).

Results

Comparative Genomic Hybridisation

In the 6 flat adenomas, on average 3.2 (range 0-4) losses and 1.8 gains (range 1-4) were found. In the 12 flat carcinomas the mean number of losses and gains were 3.5 (range 1-6) and 4.5 (range 0-8), respectively. Most frequent alterations in the flat adenomas (Figure 1) were loss of 18q (83%), 17p (50%) and 18p (50%), and gain of 20q (83%) and 20p (50%). One case had a high-level amplification on 20q13 and another case had a high-level amplification on 13q33-34. In the flat carcinomas (Figure 2) loss of 18q (92%), 4q (58%) and 4p (42%), and gain of 20q (92%), 20p (58%), 7p (42%), 7q (42%) and 17q (42%) were most frequent. In two of these cases high-level amplifications on 20q13 were found.

The set of 194 lesions from the previous study consisted of 112 adenomas and 82 carcinomas. Hierarchical clustering of the 112 polyploid adenomas had resulted in 4 clusters, and for the 82 polyploid carcinomas in 2 clusters [10]. One of the four adenoma clusters and one of the two carcinoma clusters were marked by high frequencies of 18q12-21 loss in combination with 20q13 gain. The flat adenomas and carcinomas of the present study harboured in almost all cases exactly these two aberrations. Chromosomal losses at 17p and gains at 8q and 13q, common in other subgroups of polyploid adenomas and carcinomas were considerably less frequent in the flat lesions (Figure 3).

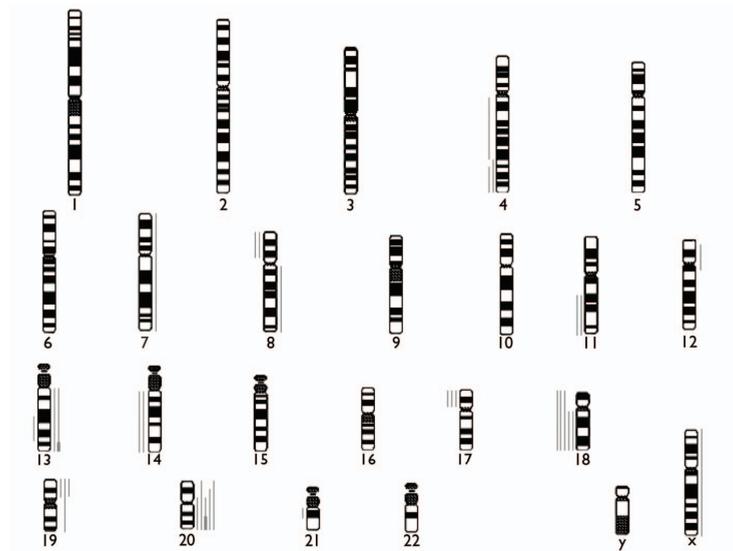


Figure 1. Overview of CGH results in 6 flat colorectal adenomas. The most striking chromosomal aberrations were loss of 18q and gain of 20q, which appeared in almost all cases (5/6). Two high level amplifications (thick bars) were found, on 13q33-34 and on 20q13, respectively.



Figure 2. Overview of CGH results in 12 flat carcinomas. Many chromosomal aberrations occurred, but loss of 18q and gain of 20q appeared in almost all cases (11/12). On chromosome 20q13, two high level amplifications (thick bars) were found.

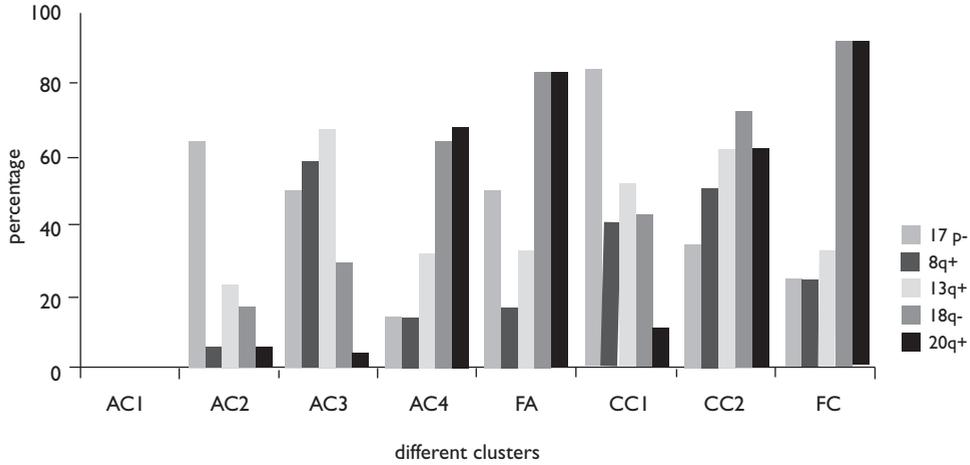


Figure 3. Chromosomal aberrations in flat colorectal adenomas and carcinomas in comparison to their distribution in different clusters of polyploid colorectal tumours, previously reported (see reference 10). Adenoma cluster 1 (AC1) had no aberrations, the other polyploid adenoma clusters were characterized by 17p loss (AC2), 8q and 13q gain (AC3), and by 18q loss and 20q gain (AC4). Clustering of the polyploid carcinomas yielded two subgroups, one showing a high frequency of 17p loss and a low frequency of 18q loss and 20q gain (CCI), and the other with a reverse pattern (CC2). The high frequency of 18q loss and 20q gain in flat colorectal adenoma (FA) and carcinoma (FC) resembles the pattern of chromosomal aberrations in the adenoma cluster AC4 and carcinoma cluster CC2.

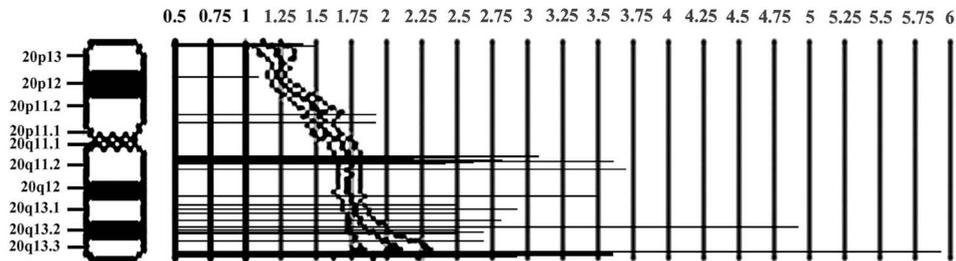


Figure 4. CGH and MLPA results of a flat carcinoma (F8) with a high level amplification on 20q13. The CGH result is shown as a continuous line paralleled by 2 lines representing the 95% confidence interval. The MLPA results are shown as horizontal bars. The vertical lines (0.5 - 6) represent the scale for copy number ratios (ratio 1 means no change). The CGH and MLPA results show a 20q amplification, however the DNA copy number ratios obtained with MLPA were higher than in the CGH analysis.

Multiplex Ligation-dependent Probe Amplification.

With CGH, gains on 20q occurred in both flat adenomas and carcinomas in a high frequency (83% and 92%, respectively) and in three cases high-level amplifications on 20q13 were found. To compare the 20q aberrations in the flat tumours to those in polypoid colorectal tumours, we used the 25 probe set as described in materials and methods. The MLPA and CGH yielded highly concordant results, however the DNA copy number ratios obtained with MLPA analysis were in most of the experiments higher than in the CGH analysis (Figure 4). Almost all tumours analysed showed gains for most of the chromosome 20 genes in the probe set (Figure 5a), except for one flat tumour (F7) that only showed gain in the 20q11 region. Both in flat and polypoid tumours, *BCL2L1*, *E2F1*, *TOP1* (median ratios of 2.0 or higher) and *ZNF217* (median ratio around 3) showed the highest copy numbers (Table 1). Comparison of flat and polypoid tumours yielded no further differences (Figure 5b).

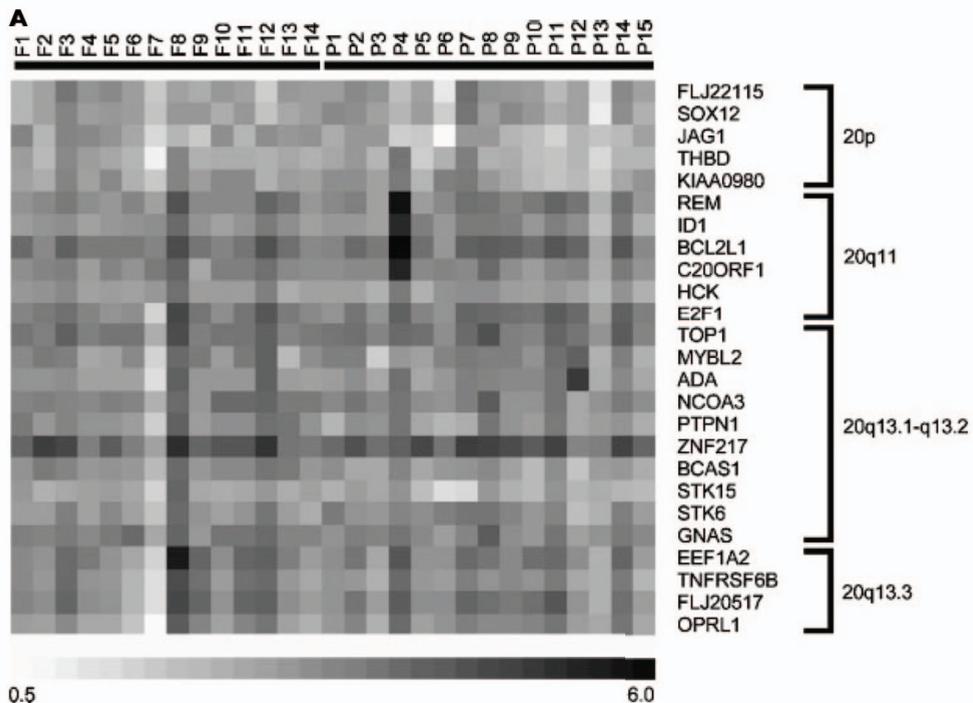


Figure 5 (A). A heatmap of the MLPA results for 25 genes on chromosome 20 of 14 flat (F1-F14) and 15 polypoid (P1-P15) colorectal adenomas and carcinomas. Rows are genes and columns are tumours. The grey level of every square represents the copy number ratio of an individual gene in an individual tumour. Both flat and polypoid tumours showed a similar pattern of DNA copy number ratios with almost all genes on chromosome 20 gained. *BCL2L1*, *E2F1*, *TOP1* and *ZNF217* were amplified, and had median copy number ratios of 2.0 or higher. *ZNF217* even had a median ratio around 3.0.

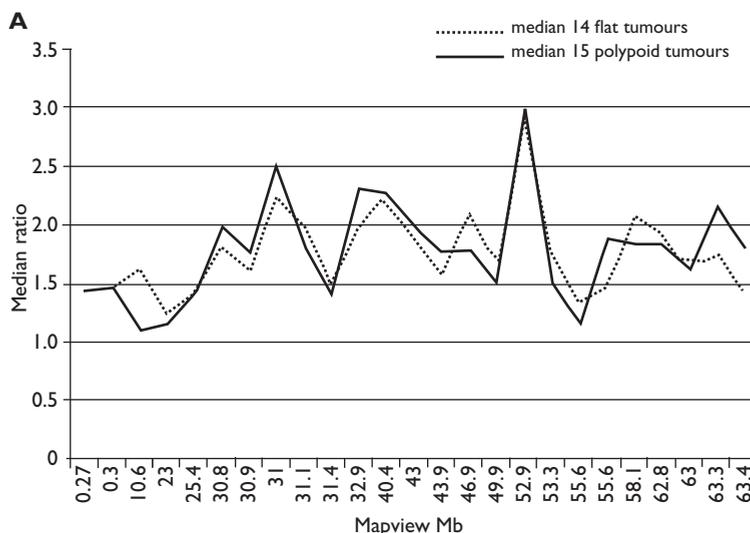


Figure 5 (B) MLPA based copy number profiles of chromosome 20 in flat (dashed line) and polyploid (solid line) colorectal tumours show similar patterns.

Table 1. Median and range of relative DNA copy number ratios for *BCL2L1*, *E2F1*, *TOP1*, and *ZNF217* in flat and polyploid colorectal tumours.

Gene	Flat tumors (n=14)	Polyploid tumors (n=15)
<i>BCL2L1</i>	2.2 (1.9-3.6)	2.5 (1.3-7.6)
<i>E2F1</i>	2.0 (0.8-3.7)	2.3 (1.4-2.9)
<i>TOP1</i>	2.2 (1.2-3.5)	2.3 (1.6-3.3)
<i>ZNF217</i>	2.9 (1.4-4.9)	3.0 (1.9-4.2)

Discussion

Due to a higher awareness among gastroenterologists and improved resolution of colonoscopes, flat adenomas of the colon are increasingly found. It has been suggested that these flat lesions, which are often small yet more severely dysplastic than polypoid adenomas of the same size, follow a different genetic pathway than the conventional polypoid adenomas [13-18]. However, for a considerable period of time, these pathways have not been defined in great detail. A recent study of chromosomal copy number changes in polypoid colorectal tumours, has demonstrated the existence of multiple routes within the chromosomal instability pathway, the major genomic pathway to colorectal cancer. The present study suggests that in fact flat lesions follow a similar genetic pathway as a particular subgroup of polypoid tumours. This subgroup of tumours is marked by a

high frequency of 18q12-21 loss in combination with 20q13 gain. Presence of these chromosomal aberrations correlates with higher grades of dysplasia and higher risk of progression to colorectal cancer, and therefore this category could represent a more aggressive subclass of adenomas [10]. Interestingly, also in the flat adenomas 18q loss and 20q gain occurred very frequently (both 83%), which fits well with the observation that flat adenomas behave more aggressively [5,19-21]. Recently Richter et al. also compared chromosomal imbalances in flat and polypoid colorectal tumours [22]. Like in the present study, a higher frequency of 18q losses in flat carcinomas was found. In several other aspects, both studies are less easy to compare. Richter et al. studied mainly low-grade dysplasia adenomas, whereas all adenomas in the present study showed high-grade dysplasia. This is even more relevant since the distinct differences between flat and polypoid lesions in the series of Richter et al. were mainly found in these LGD adenomas. Yet, these aberrations occurred in low frequencies (gains of 2q(3/22), 5q(2/22), 6(2/22), 8q(2/22) and 12q(2/22), and losses of 17p(5/22) and 20(3/22)), whereas we have focussed on chromosomal aberrations that in colorectal tumours overall occurred with frequencies >40% [10]. Chromosome 16p, loss of which by Richter et al. was reported as another major difference between flat and polypoid lesions, has been excluded from analysis in all our CGH studies because, like chromosomes 1p32-pter, 19 and 22, it has a high content of repetitive DNA, which may interfere with the results of chromosome based CGH [23]. The high frequency of 20q gains in the flat lesions in the present study, in contrast to the findings of Richter et al., was confirmed by the MLPA findings. Also this could be caused by the different compositions of both series.

The genes involved in 18q12-21 loss and 20q13 gain are not completely known, but given the high correlation between these copy number changes it is likely that they have a synergistic effect in deregulating cellular pathways that are critical for tumour development. On chromosome 18q21, the *DPC4* (*Smad4*) gene is a likely candidate. *Smad4* is a key factor in the TGF β signalling pathway and plays an important role in colorectal cancer progression. In polypoid adenomas that were initiated by loss of *APC*, the mere inactivation of *Smad4* was able to induce malignant transformation [24]. What the additional role of gain of 20q would be is not exactly clear. Amplification at 20q13 occurs in a variety of tumour types, including breast, colorectal and gastric cancer [25,26], and is associated with aggressive behaviour.

To further pinpoint DNA copy number gains on 20q in colorectal tumours to the level of individual genes, we used MLPA with a dedicated probe set containing 25 probes on chromosome 20, twenty of which covered three different regions on 20q, i.e. 20q11, 20q13.1-13.2 and 20q13.3. With this approach we could demonstrate that also at high resolution no differences could be found between the 20q gains in flat colorectal tumours and the subgroup of polypoid colorectal tumours

with 20q gain. Four of the twenty 20q probes tested, i.e. *TOP1*, *BCL2L1*, *E2F1*, and *ZNF217*, were amplified with median DNA copy number ratios of 2.0 or higher. Of these, *ZNF217* even had a median ratio around 3.0. Unfortunately, no further material of these tumours was available for checking the RNA expression of these genes.

ZNF217 located at 20q13.2 is predicted to encode alternately spliced, Kruppel-like transcription factors of 1,062 and 1,108 amino acids, each having a DNA-binding domain (eight C2H2 zinc fingers) and a proline-rich transcription activation domain [27]. *ZNF217* encodes a transcription factor and is overexpressed in breast cancer [27-29]. Overexpression of *ZNF217* in human mammary epithelial cells results in immortalisation and overcoming senescence [29]. Amplifications of *ZNF217* have also been detected in ovarian cancers [30,31], and increased copy numbers of this gene were significantly associated with reduced survival in patients with ovarian cancer [32]. In colorectal cancer, amplification of *ZNF217* has been associated with increased metastatic potential [33].

TOP1, located at 20q12-13.1, encodes DNA topoisomerase I. This enzyme controls and alters the topologic states of DNA during transcription. DNA topoisomerase I catalyses the transient breaking and rejoining of a single strand of DNA which allows the strands to pass through one another, thus altering DNA topology. Formation of a DNA-topoisomerase I complex is a crucial intermediate step in the relaxation of DNA; however, the complex is potentially dangerous to the cell, because it can mediate illegitimate recombination that may lead to genomic instability and oncogenesis [34]. Zhao et al. demonstrated that elevated expression of *TOP1* is correlated with poor disease-free survival in patients with breast tumours [35]. An intriguing point is the relation between *TOP1* amplifications and treatment with topoisomerase I inhibitors, like irinotecan. Given the fact that none of the patients had received neoadjuvant chemotherapy, the *TOP1* amplifications in these tumours can't be therapy induced. How, on the other hand, the presence of *TOP1* amplifications would affect the response to topoisomerase I inhibitors is another important point, but this lies beyond the scope of the present paper.

However, not only the 20q12-13 region seems important, but also 20q11.21 where *BCL2L1* is located. *BCL2L1*, also known as *Bcl-X*, belongs to the *Bcl-2* family. *Bcl-2* family members form hetero- or homodimers and act as anti- or pro-apoptotic regulators. *BCL2L1* proteins regulate outer mitochondrial membrane channel (VDAC) opening [36], which in turn regulates mitochondrial membrane potential, and thus controls the production of reactive oxygen species and release of cytochrome c by mitochondria, both of which are potent inducers of programmed cell death. Two *Bcl-X* splice variants exist, the larger *Bcl-X* (*Bcl-XL*) acts as a repressor of programmed cell death and the smaller splicing product (*Bcl-XS*) as an apoptotic activator. Overexpression of the *Bcl-XL* protein has been reported in >60% of human colon cancers [37,38].

E2F1, located at 20q11.2, is a member of the E2F family of transcription factors which regulates G1 to S phase progression. The E2F1 protein is inactivated by binding to retinoblastoma protein (pRB). D-type cyclins and cyclin E, acting together with cyclin-dependent kinases, phosphorylate the retinoblastoma protein (pRb), which results in the activation of E2F1, and consequently drive the G1 to S phase transition [39]. Thus *E2F1* can mediate cell proliferation and is tightly associated with oncogenesis [40].

We conclude that the pattern of chromosomal instability in flat colorectal adenomas and carcinomas is marked by a high prevalence of 18q loss and 20q gain, resembling one of the distinct patterns of chromosomal changes found in polypoid colorectal adenomas and carcinomas, and that abnormalities in these two chromosomal regions could convey a more aggressive clinical behaviour. Based on the level of copy number changes and their putative functions, *ZNF217*, *TOP1*, *BCL2L1* and *E2F1* are important candidate genes for driving the 20q gain in colorectal cancer.

Acknowledgements

This work was supported by the Dutch Cancer Society grants KWF-VU 97-1455 and KWF-VU 02-2618.

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