Molecular discrimination of sessile rectal adenomas from carcinomas for a better treatment choice: integration of chromosomal instability patterns and expression array analysis

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CHAPTER 4

Single nucleotide polymorphism array analysis of chromosomal instability patterns discriminates rectal adenomas from carcinomas

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Abstract

Total mesorectal excision (TME) is the standard treatment for rectal cancer, while transanal endoscopic microsurgery (TEM) is a recently introduced surgical approach for the treatment of rectal adenomas. Incorrect preoperative staging before TEM is a problem. To identify genetic changes that might correlate with tumour stage and could lead to optimized treatment selection we performed a genome-wide chromosomal instability search in a homogeneous, clinical cohort of rectal tumours. 78 rectal tumours during different clinical stages were analysed with 10K single nucleotide polymorphism (SNP) arrays. Logistic regression was performed to build a quantitative model of specific chromosomal aberrations. Overall, most cases (95%) had one or more chromosomal aberrations. We observed a clear correlation between the total number of aberrations and the different tumour stages. Specifically, the chromosomal events: gain of 8q22-24, 13q and 20q, and loss of 17p and 18q12-22, were far more abundant in carcinoma than in adenoma. In adenoma fractions from cases with a carcinoma (infiltrating at least in the submucosa), twice the amount of such ‘malignant aberrations’ was observed, compared to pure adenomas. Furthermore, combined aberrations such as gain of 13q and loss of 18q were only found in adenomatous fractions of carcinomas and not in benign lesions. Based on these five genomic events associated with carcinoma, a clear distinction between adenoma and carcinoma tissue could be made. These data should be validated further in order that they may be used in preoperative staging of rectal tumours.

Introduction

Total mesorectal excision (TME) and preoperative radiation is the standard treatment for rectal cancer in most North and West European countries (1). However, this treatment results in significant functional morbidity (2). Recently, transanal endoscopic microsurgery (TEM) has been developed. This sparing technique for the local resection of rectal adenomas results in minimal mortality and morbidity (3-5). In spite of adequate preoperative staging, carcinomas (invasive in at least the submucosa) can be found after TEM treatment, with an increased risk of local recurrence and lymph node metastases. Reported risks of local recurrence vary considerably: between 10 and 67% (reviewed in (4, 6-10)), with more recent studies reporting lower risks between 4.3% and 11% (11-14). Recent experimental evidence states that local treatment of T1 carcinomas by TEM may be possible (10, 14). As T1 rectal tumours have a chance of 12% of lymph node metastasis (15), preoperative knowledge on the aggressive behaviour of a tumour is important.

One of the characteristics of human cancer is chromosomal instability (16). For rectal cancer, this is the predominant characteristic (17). The common patterns of chromosomal instability in colorectal cancer include gains on chromosomes 13q and 20q, loss of 17p and 18q and loss of heterozygosity (LOH) of chromosomes 5q, 8p, 17p and 18q (18-24). The patterns of chromosomal losses and gains, and copy number neutral LOH can accurately and simultaneously be studied from the same sample with single nucleotide polymorphism (SNP) arrays (25-27). This combined analysis offers the advantage of detecting extra genomic events, which would have been disregarded with other techniques (28, 29). Combined with standard clinicopathologic variables, these chromosomal aberrations could serve as accurate biomarkers (30, 31) and improve preoperative staging of rectal tumours. For colorectal cancer, associations were found between prognosis and changes of chromosomes 8 and 18q (30); between high-grade dysplasia and gain of chromosome 7 and 20 and loss on 17p and 18q (31) and gain of chromosome 8q23 with lymph node metastases (32). However, owing to methodological differences and heterogeneity in study populations, there is no conclusive evidence on the prognostic significance of commonly implicated regions (33). Furthermore, left- and right-sided colon cancer clearly differ in their aetiology, clinical behaviour, pathological features and genetic abnormalities (34-38).

To mark the progression from adenoma to carcinoma in a homogeneous clinical cohort of rectal tumours, we studied genome-wide copy number changes and LOH using SNP arrays. After extensive validation, the identified patterns of aberrations might complement the current criteria for treatment selection.

Material and Methods

Samples

Seventy eight snap frozen rectal adenomas and carcinomas were collected. 43 samples from patients from the IJsselland Hospital or Reinier de Graaf Hospital in The Netherlands who had undergone TEM treatment and 35 samples from a Dutch multicentre TME trial were included (1). None of these patients received radiotherapy or other adjuvant therapy. Leukocyte DNA was available for 11 cases. All samples were reviewed by a pathologist (H.M.), dysplasia was scored, and the tumour-percentage was assessed (50-80%). Intramucosal carcinoma were considered as adenomas with high-grade dysplasia, instead of invasive carcinomas (39, 40). The local medical ethics committee approved the study.

Copy number and LOH analysis

Tumours were microdissected in a cryostat through removal of surrounding healthy tissue. Twenty 30-µm sections were cut from each tumour. To guide microdissection, a 4-µm section was cut and haematoxylin and eosin (H&E) stained, before the first section, and after the tenth and twentieth section, and assessed for the presence of adenoma or carcinoma tissue or a mixture of both. DNA was isolated with the Genomic Wizard kit, according to the manufacturer (Promega, Madison, WI, USA). Leucocyte DNA was obtained as described (41). DNA quality was checked on a 1% agarose gel.

DNA from leucocytes and fresh frozen tumours was hybridized to GeneChip Mapping 10K 2.0 arrays (Affymetrix, Inc., Santa Clara, CA, USA) at the Leiden Genome Technology Centre (http://www.lgtc.nl), as previously described (42). Genotypes were scored with the GDAS software (Affymetrix).

For normalization we used in-house normal reference samples consisting of 11 leucocyte DNAs from TEM samples and 11 unrelated controls. Copy number and LOH data were calculated as follows:

a) Cell files of the tumour samples were normalized with the 22 reference samples and analysed in dCHIP SNP (43). Raw copy number data were exported and smoothed with an R-script computing a median curve and lower and upper quartile curves that indicate the spread of the data (44). When this 50% interval fell entirely under the n=2 diploid baseline, it was called a loss and when it fell above this line it was called a gain. LOH calls were obtained with the Hidden Markov Model, which was implemented in dCHIP SNP.
b) Additionally, non-allele specific analysis was performed in the Copy Number Analyzer for Affymetrix GeneChip Mapping arrays (CNAG) (45). The best combination of normal references was computed by CNAG.

c) Allele-specific analysis with CNAG was performed for the 11 TEM cases with corresponding leucocyte DNA as described (45).

All data were imported in an Access database (Microsoft, Redmond, WA, USA) and the average value per chromosome sub-band was calculated. Average size of a chromosome sub-band was 2.96 Mb (range 0.002-15.02 Mb). Only aberrant sub-band regions identified by all analysis methods were considered and three different events were discerned: loss, gain, and copy number neutral LOH.

Array comparative genomic hybridization (array-CGH)

Array-CGH was performed for four control samples, including three from a previous study (46) as previously described (47).

Illumina SNP arrays

We used Illumina SNP arrays, which are suitable for LOH detection and copy number analysis of formalin-fixed, paraffin-embedded (FFPE) tissue (46, 48). Briefly, DNA extracted from FFPE tissue samples was prepared according to the Goldengate assay in combination with linkage mapping panel version 4 and hybridized to Illumina BeadArrays. Copy number aberrations and LOH were determined using the in house generated "beadarraySNP" R-package.

Statistics

One way ANOVA and Bonferroni *post hoc* tests were performed on the square root of the number of aberrations in order to stabilize the variance. Chi^2 tests were performed to test significance between groups for specific loss and gain events. Physical loss and copy number neutral LOH were considered as identical events in this analysis. For all analyses, p-values <0.05 were considered significant. All analyses were performed in SPSS 12.

For logistic regression analysis, a model was built in Matlab, in which each chromosome and each group is characterized by specific odds of occurrence of events. The model is $\gamma_{ct} = \alpha_c \beta_t$, where γ_{ct} indicates the odds for the combination of chromosome c and tumour group t, α_c the odds for chromosome c, and β_t the odds for tumour group t. This model supposes that there is no interaction between chromosomes and groups. It also assumes that, if the odds for one chromosome (over the groups) are known, the odds for other chromosomes, and similarly groups (over the chromosomes), are known as well. The use of logarithms ($a_c = log\ \alpha_c$ and $b_t = log\ \beta_t$) leads to an additive model for the log-odds, which can be fitted through logistic regression.

Results

Sample description

Genomic DNA from 78 snap frozen rectal tumour samples from 77 different patients was hybridized to 10K SNP arrays in order to determine copy number changes and allelic imbalances. The samples analysed consisted of adenomas and carcinomas. Subsequently, five subcategories were defined on the basis of tissue fraction analysed and tumour stage (Table 1 and Supplementary Table 1). The adenomas were subdivided into cases consisting of only adenoma tissue in the resection (A/A) and adenoma fractions of cases with a carcinoma focus infiltrating at least in the submucosa (A/C). The carcinomas were subdivided into three groups: tumour fractions consisting of a mixture of adenoma and carcinoma tissue (AC/C), carcinomas without (C/C), and carcinomas with lymph node metastasis (C/C (N+)). In a single case, A140, both the adenoma and carcinoma fractions were analysed separately.

SNP array data analysis and validation by array-CGH

Copy number and LOH profiles from the SNP array data were generated with two algorithms, dChip SNP and CNAG, and compared with array-CGH in four control cases. Figure 1 shows an example. Physical loss of chromosome 4q is detected by quantile smoothing of dCHIP data, CNAG raw and smoothed data, and array-CGH (Figure 1A-C).

Table 1. Summary of clinical and pathological data of 78 tumour samples

	A/A	A/C	AC/C	C/C	C/C(N+)
Treatment					
TEM	21	8	7	5	2
TME	2	3	2	17	11
Sex (M/F)	12/11	5/6	5/4	10/12	10/3
Age (mean)	69	70	66	65	62
Dysplasia (adenoma)					
low	12	7			
high	11	4			
Stage (carcinoma)					
T1		8	4	10	
T2		4	4	12	12
T3			1		1
Size(cm) (mean)	6.2	4.8	4.2	3.1	5.3

A/A adenomas; A/C tumours consisting of adenoma and carcinoma tissue, from which we analyzed the adenoma fraction; AC/C tumours consisting of adenoma and carcinoma tissue, from which we analyzed a mixture of adenoma and carcinoma tissue; C/C carcinomas without lymph node metastasis; C/C(N+) primary tumours of cases with lymph node metastasis.

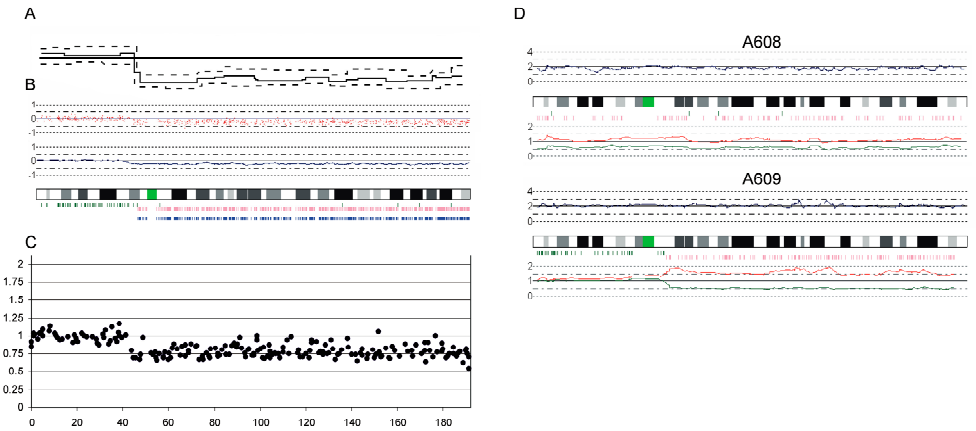

Figure 1. A-C. Chromosome 4 for tumor A514. A. Smoothed trend computed with dCHIP SNP data and quantile smoothing algorithm. Solid line represents the median, dashed lines represent 25% and 75% quantiles. B. Upper panel shows raw copy number estimates by CNAG, while lower panel shows smoothed copy number. Stripes under the chromosome display heterozygous (green), homozygous SNPs (pink) and SNPs showing LOH (blue). C. ArrayCGH image for verification. D. Allele specific analysis with CNAG. Chromosome 5 is shown for tumour A608 (top) and A609 (bottom). The blue line indicates smoothed copy number, while the green and red lines indicate the two alleles. The pink stripes under the chromosome indicate LOH.

Allele specific analysis shows two different LOH mechanisms: one allele of chromosome 5q is lost in case A608, while the other is retained, indicating LOH due to physical loss. In contrast, one allele was deleted from sample A609, while the other allele was amplified, resulting in copy number neutral LOH (Fig. 1D). In the four control cases, all gains and physical losses that were identified with the SNP array analysis were concordant with array-CGH data. Additionally the SNP arrays identified regions of copy number neutral LOH on chromosome 5q, 9, 12 and 17p.

Number of chromosomal aberrations in different groups

An overview of the extent of chromosomal instability in all different tumour samples was generated. Therefore, the number of aberrant chromosome sub-bands per sample was counted. All but four of 78 cases showed one or more chromosomal aberrations (95%) (Supplementary Table 1 and Figure 2). Overall, an average for each sample of 65 sub-bands showing physical loss, 81 sub-bands showing gain, and 29 sub-bands showing copy number neutral LOH was detected. A significant increase (ANOVA, $p<0.001$) in aberrations was

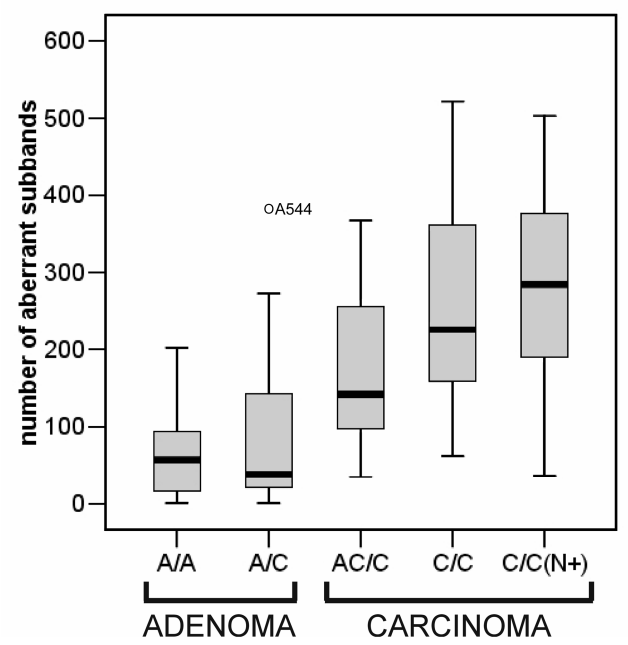

Figure 2. Box plot of mean number of aberrant chromosome sub-bands per group. Median, 25th and 75th percentiles, and range of expression levels are shown.

found for the more aggressive tumours. Significant differences were found when adenoma tissue (A/A and A/C) was compared with carcinoma tissue (C/C and C/C (N+)) (68 and 100, respectively, versus 281 and 290 mean number of aberrations, $p<0.001$).

Chromosomal aberrations in adenoma versus carcinoma tissue

For several aberrations, the minimal region of overlap was listed, although in most cases whole chromosome arms were involved. Figure 3 displays all the gains, physical losses and copy number neutral LOH events for each sample. Table 2 summarizes the most common events. Physical loss and copy number neutral LOH events are combined in this table. Loss of 1p36 (26%), 4q32-ter (26%), 5q (30%), and gain of 7p11-15 (26%), 12q13 (22%) were frequently observed aberrations in the adenomas. As the occurrence of these events had not significantly increased in carcinoma, they are indicated as early events (loss of 1p36, loss of 4q32-ter, loss of 5q, and gain of 12q13). There were no significant differences in frequency of these events between low-grade and high-grade dysplastic adenomas (data not shown). Gain of 7p11-15 was increased in carcinomas ($p = 0.005$). However, gain of 8q22-24 (50%), 13q (59%), 20q (86%) and loss of 17p (91%), 18q12-22 (86%) were the most frequent carcinoma aberrations. Percentages of these aberrations were all significantly

higher in the carcinomas than in the adenomas (p<0.001). Therefore, they were called carcinoma or malignant events. Gain of 1q23 was observed to be more common in carcinomas with lymph node metastases (62%) than in carcinomas without lymph node metastases (14 %) (p=0.003).

Frozen adenoma tissue from A/A cases was compared with frozen adenoma tissue from A/C cases in order to determine if carcinoma events were more common in the adenomas of cases with a carcinoma focus than in pure adenomas. Gain of 13q was such an event, which was only detected in one A/A case (out of 23), compared to four (out of 12) A/C cases (p=0.017). A frequency table of all different combinations of chromosomal events in the different adenoma and carcinoma subgroups was created (Supplementary Table 2). Gain of 13q in combination with 18q loss showed the highest frequency in the A/C group (27%), while it did not occur in the A/A cases (p=0.007) (Table 2). All other

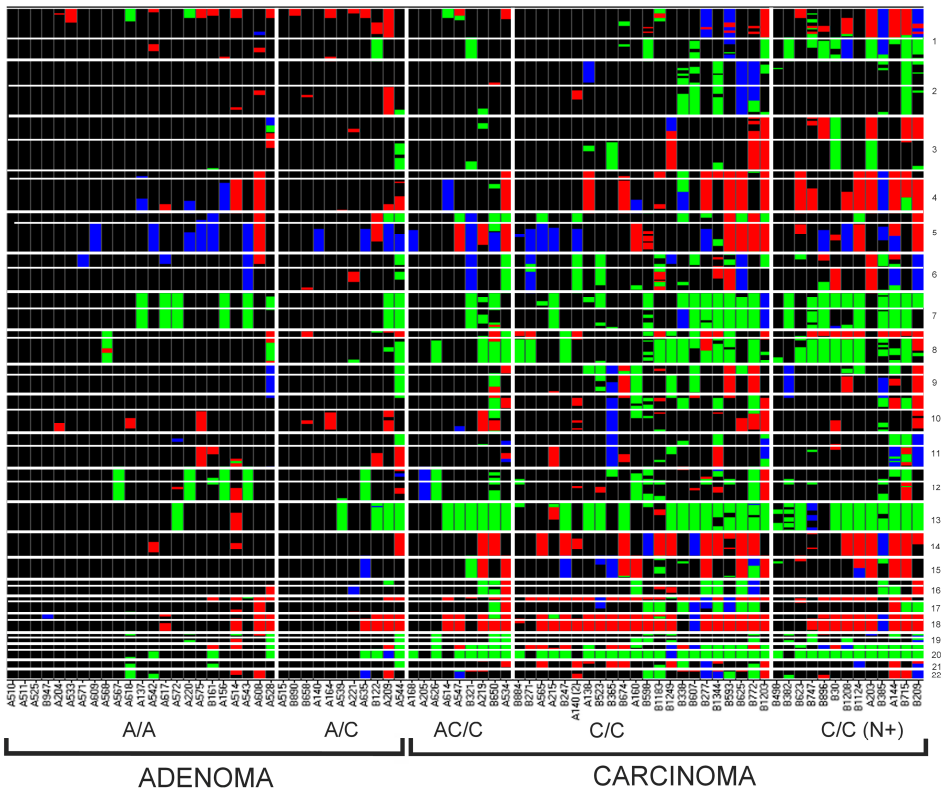

Figure 3. Overview of all gains (green), physical losses (red) and copy number neutral LOH (blue) in 78 rectal tumour samples. On the x-axis are all the different samples in the five different subgroups. On the y-axis are the chromosomes. The thick horizontal lines represent chromosomal borders; the thin lines represent centromere position in the meta-centric chromosomes.

Table 2. The most common chromosomal aberrations (%).

	ADENOMA		CARCINOMA			P-VALUE*		
	A/A (n=23)	A/C (n=11)	AC/C (n=9)	C/C (n=22)	C/C(N+) (n=13)	Adenoma vs. Carcinoma	A/A vs. A/C	C/C vs C/C(N+)
early events								
loss 1p36‡	26	45	22	14	46	n.s.	n.s.	0.035
loss 4q32-qter‡	26	27	22	36	62	n.s.	n.s.	n.s.
loss 5q┼	30	45	67	55	54	n.s.	n.s.	n.s.
gain 7p15-11	26	18	33	50	77	0.005	n.s.	n.s.
gain 12q13	22	9	22	32	23	n.s.	n.s.	n.s.
carcinoma events								
gain 8q22-24	9	18	44	50	62	<0.001	n.s.	n.s.
gain 13q	4	36	67	59	85	<0.001	0.017	n.s.
loss 17p‡	17	18	44	91	62	<0.001	n.s.	0.038
loss 18q12-22‡	17	36	56	86	77	<0.001	n.s.	n.s.
gain 20q	17	27	78	86	92	<0.001	n.s.	n.s.
gain 13q combined with loss 18q12-22	0	27	56	59	62	<0.001	0.007	n.s.
associated with lymph node metastasis								
gain 1q23	0	9	11	14	62	0.002	n.s.	0.003

* p-values were computed by Chi^2 test

‡ most cases showed physical loss and LOH, while some showed copy number neutral LOH

┼ most cases showed copy number neutral LOH, while some showed physical loss and LOH

combinations were more frequent in the carcinoma groups than in the adenoma groups, e.g. 18q loss in combination with 20q gain (4% in the A/A vs. 27% ranging to 80% in the different carcinoma groups).

Quantitative progression model

Loss of 17p and 18q12-22 and gain of 8q22-24, 13q and 20q were the most frequent events in carcinomas, in contrast to adenomas (all with p-value < 0.001(Figure 3 and Table 2)). Therefore, these five events were used to build a quantitative progression model from adenoma to carcinoma. Figure 4A summarizes the observed frequencies of these five events in the different sample subgroups. The log-odds for each event and each sample group were calculated and fitted in a model through logistic regression analysis (Figure 4B an C). There was a strong correlation between the observed frequencies and the model fit (r=0.95). The gradual increase of these events in the subgroups indicates that these five aberrations are highly correlated with the adenoma to carcinoma progression in rectal tumorigenesis.

Comparison of adenoma and carcinoma fractions of single cases

The frozen tissue contained sufficiently large adenoma and carcinoma fractions to be analyzed separately in only a single case (A140). The remaining A/C cases showed large adenoma fractions with relatively small carcinoma foci that were not obtained during the procedure of snap freezing of the tumour material. For those cases categorized as C/C and C/C (N+) macroscopy was quite different, as they contained no or hardly any precursor

adenoma material. As we had recently demonstrated reliable LOH and copy number analysis in FFPE material using Illumina SNP Arrays(46, 48), we analysed FFPE-derived tissue fractions from 16 cases (including case A140) and compared adenoma (either low- or high grade dysplasia) and carcinoma tissue (Figure 5). The results were compared with those from the frozen tissue samples. With relatively minor differences the chromosomal aberrations in the corresponding tumour fractions were comparable. In 11 cases one or more 'malignant aberrations' (8q22-24 gain, 13q gain, 17p loss, 18q12-22 loss, 20q gain) were detected in the adenoma fractions. In sample A138 even four 'malignant aberrations' were detected in the adenoma fraction with low-grade dysplasia. The progression of adenoma to carcinoma was in 11 out of 16 cases characterized by one to three extra 'malignant aberrations' (three cases 8q gain, six cases 13q gain, four cases 17p loss, six cases 18q loss and four cases 20q gain). Five cases showed a comparable amount of aberrations in their adenoma and carcinoma fractions respectively.

Discussion

After TEM treatment 10-30 % of presumed rectal adenomas prove to be carcinomas, infiltrating at least into the submucosa (tumour stage T1 and further). Since the introduction of preoperative endorectal ultrasound this figure has declined to 10% (10), but there is still a need for better preoperative parameters to discriminate rectal adenomas from carcinomas.

We used SNP array analysis and identified that gain of 8q22-24, 13q, 20q and loss of 17p and 18q12-22 are more prevalent in rectal carcinomas than in adenomas. Several other studies in colorectal cancer have also identified these regions (18, 20-24, 31). Hermsen *et*

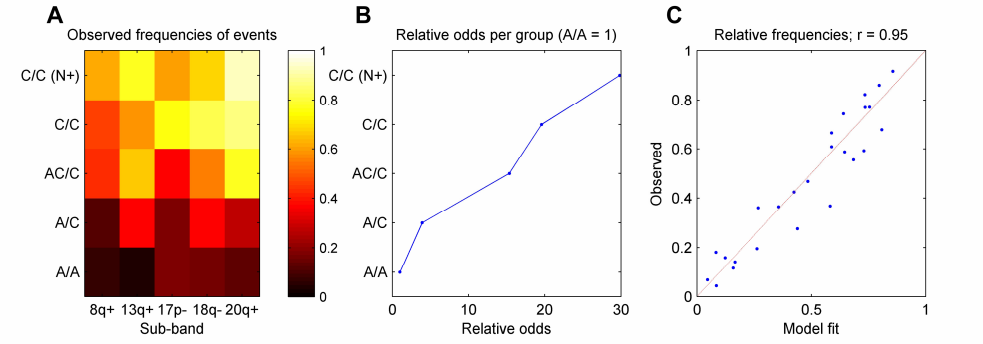

Figure 4. Logistic regression analysis. The observed frequency of the specific events is shown as a colour scale (A), the relative odds per group (B), and the model fit (C).

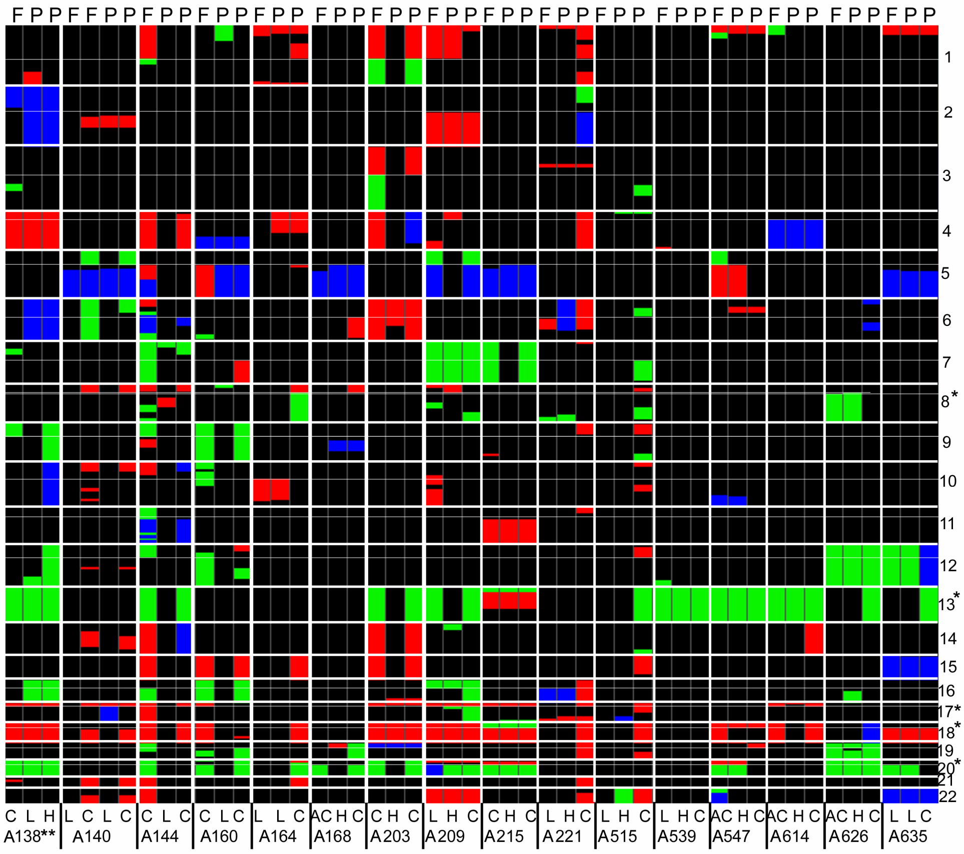

Figure 5. Overview of gains (green), physical losses (red) and copy number neutral LOH (blue) in adenoma and carcinoma fractions of 16 rectal tumour samples. On the x-axis are all the different samples, successively the frozen sample and the FFPE- adenoma and -carcinoma fraction. On the y-axis are the chromosomes. The five 'malignant aberrations' are indicated by an asterisk (*). Top of figure: F (frozen), P (paraffin-embedded). Bottom of figure: L (adenoma with low grade dysplasia), H (adenoma with high grade dysplasia), AC (mixture of adenoma and carcinoma), C (carcinoma). The carcinoma fraction in the FFPE tissue from sample A138 was too small to analyse (**).

al. showed that two or more of seven specific chromosomal regions (loss on 8p, 15q, 17p, 18q, and gain on 8q, 13q and 20q) were associated with colorectal cancer progression (20). Leslie *et al.* have identified that chromosomal loss on 17p, and 18q and gain of 20were related to the onset of high-grade dysplasia (31). Diep *et al.* have conducted a meta-analysis from 31 CGH studies and found that losses at 17p and 18 and gains of 8q, 13q, and 20

occur early in primary colorectal cancers (18). Hence, the regions found in our study are highly similar to those found in other studies; although part of the events identified would have been missed by CGH, owing to copy number neutral LOH (28, 29). Subsequently, logistic regression analysis was performed, and these five events together were shown to build a quantitative progression model of rectal tumourigenesis.

We found that rectal adenomas showed relatively few aberrations, while carcinomas showed many. However, exceptions were identified: adenomas with many aberrations and carcinomas with few aberrations. In adenoma fractions from cases with a carcinoma, twice the amount of such 'malignant aberrations' was observed, compared with pure adenomas. Furthermore, comparison of adenoma and carcinoma tissue from the same lesion, showed in the majority of cases (11/16) an increase of one to three of the 'malignant aberrations' in the carcinoma fraction. By comparing carcinomas with and without lymph node metastases, we discovered that lymph node positive cases demonstrated an amount of chromosomal aberrations that was similar to that in the lymph node negative cases. This indicates that genomic instability is an early event in the progression of rectal cancer, as has also been shown by others (reviewed in (49)). However, one remarkable difference was found: cases with lymph node metastasis more frequently showed gain on 1q. In other studies a 1q gain has been related to metastases to the liver, but not to the lymph nodes (18, 50). Another study found a strong correlation between 8q23-24 gain and lymph node metastases (32). However, we detected equal percentages of 8q gain in the carcinoma with and without lymph node metastases. The gain on 1q and a possible relation to lymph node metastasis needs further validation in a larger series. Currently, expression array analysis is performed on the same samples, and both data types are integrated. These combined data might reveal novel markers for lymph node metastasis.

A strength of this study concerns the homogeneity of the sample collection. All samples were obtained from rectal tumours, while previous studies usually described a heterogeneous group of colon, rectum, and sigmoid tumours. Several recent papers have indicated differences between colonic and rectal cancer and have advocated that when prognostic markers are investigated, differences between tumour location should be considered (34, 35).

This study did not take tumour heterogeneity into account. As is known from the literature, colorectal cancers are heterogeneous (51-54). We currently study chromosomal instability in different biopsies to determine tumour heterogeneity. Initial results indicate that three biopsies reliably represent the genomic aberrations. Five out of 18 tumours showed heterogeneity, displayed by extra chromosomal aberrations in one or two biopsies.

In conclusion, a compilation of five carcinoma-specific events can accurately distinguish adenomas from carcinomas. In contrast to pure adenomas, adenoma fractions of carcinoma cases already carry 'malignant aberrations'. Such results might complement

clinical data to guide treatment selection more precisely, although thorough prospective validation in independent series is still required.

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Supplementary data

Supplementary data are available at
http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2180.html

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