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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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Citation

Lips, E. H. (2008, June 19). *Molecular discrimination of sessile rectal adenomas from carcinomas for a better treatment choice: integration of chromosomal instability patterns and expression array analysis*. Retrieved from <https://hdl.handle.net/1887/12962>

Version: Corrected Publisher's Version

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Note: To cite this publication please use the final published version (if applicable).

CHAPTER 5

**Progression and tumor heterogeneity
analysis in early rectal cancer**

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Clin Cancer Res. 2008 Feb 1;14(3):772-81

Abstract

Adequate preoperative staging of large sessile rectal tumors requires identifying adenomas that already contain an invasive focus, specifically those that are growing in or beyond the submucosa. We systematically compared chromosomal instability patterns in adenoma and carcinoma fractions of the same lesion to assess specific steps in rectal tumor progression. We analyzed 36 formalin-fixed, paraffin-embedded tumors. Both the adenoma and carcinoma fractions were typed with single nucleotide polymorphism arrays and compared with 21 previously described pure adenomas. Eighteen cases were included in an intratumor heterogeneity analysis. Five specific "malignant" events (gain of 8q, 13q and 20q and loss of 17p and 18q) and aberrant staining for p53 and SMAD4 were all increased in the adenoma fractions of carcinoma cases compared with pure adenomas. Paired analysis revealed that 31% of the samples had an equal amount of malignant aberrations in their adenoma and carcinoma fractions, whereas 25% had one and 33% had two or more extra malignant events in the carcinoma fraction. Analysis of three core biopsies per patient showed a large degree of intratumor heterogeneity. However, the number of malignant aberrations in the biopsy with the most aberrations per tumor correlated with the corresponding adenoma or carcinoma fraction ($r=0.807$; $P < 0.001$). Five specific chromosomal aberrations, combined with immunohistochemistry for p53 and SMAD4, can predict possible progression of sessile rectal adenomas to early rectal cancer and can, after validation studies, be added to preoperative staging. Preferably, three biopsies should be taken from each tumor to address intratumor heterogeneity.

Introduction

Colorectal cancer is one of the leading causes of mortality and accounts for ~300,000 new cases per year in Europe and the United States (1). Approximately 25% of these cases are rectal cancers, and the incidence of its benign precursor lesion, adenomas, is far higher. Total mesorectal excision is the gold standard to treat carcinomas (2); transanal endoscopic microsurgery (TEM) is the method of choice to treat sessile adenomas (3, 4). Although it has not yet been proven, T1 rectal carcinomas may be good candidates for TEM without compromising oncological outcome (5-7). On the other hand, an invasive carcinoma (beyond the muscularis mucosae) is found after local excision in a large proportion of presumed benign tumors (5, 8), which shows the need for more precise staging. Several possible imaging techniques have additional value, and endorectal ultrasound seems most promising; however, not all cases are eligible for endorectal ultrasound, and overstaging is a serious problem (9). It should be noted that TEM-treated cases of early rectal cancer mostly consist of adenoma tissue. Thus, there is a need for additional preoperative staging methods that can accurately facilitate therapeutic decision making in the treatment of rectal tumors. Ideally, a combination of methods should be able to reliably discern benign adenomas from adenomas containing a carcinoma focus, as well as predict lymph node metastasis.

Chromosomal instability is the main characteristic of many different tumor types, including rectal cancer. To date, many studies have been done in colorectal cancer to assess chromosomal gains, losses or LOH. Commonly involved regions in (colo)rectal cancer are 5q, 8, 13q, 17p, 18q and 20q, as established by different groups (10-14). Other studies specifically analyzed rectal cancer precursor lesions (15-18) and found that commonly involved chromosomal aberrations are already frequent in adenomas or are correlated with high grade dysplasia. Several studies identified intratumor heterogeneity, which is characterized by patterns of different chromosomal aberrations in different tumor areas of the same lesion (19, 20).

In a previous study, we used single nucleotide polymorphism (SNP) arrays to detect copy number aberrations and LOH in rectal adenomas and carcinomas at different clinical stages (12). Considering the frequent malignant events, gain of 8q, 13q and 20q, and loss of 17p and 18q, we have built a rectal cancer progression model. In addition, we found that (combinations of) these “malignant” events were increasingly found in adenoma fractions of carcinoma cases in comparison with pure adenomas. We now did a systematic comparison of chromosomal instability patterns in adenoma and carcinoma fractions in the same lesion of early rectal cancer cases that were treated by TEM. The effect of intratumor heterogeneity in a partly overlapping set of tumors was assessed by chromosomal instability analysis of three different *ex vivo* core biopsies per tumor, which were taken postoperatively.

Material and Methods

Samples

Material from 36 rectal carcinomas was obtained. These tumors were preoperatively classified as adenomas, but in all cases, definite histopathology revealed the presence of a carcinoma. All patients were treated using the TEM technique at the IJsselland Hospital (Capelle a/d IJssel, the Netherlands) or Reinier de Graaf Hospital (Delft, the Netherlands). None of the patients received (neo-) adjuvant radiotherapy or chemotherapy. All samples were reviewed by a pathologist (H.M.), dysplasia was scored, and tumor cell percentage was assessed (50-80%). From these tumors, we analyzed an adenoma (also indicated as A/C), a carcinoma (also indicated as C/C), and a normal tissue fraction. For comparison, we used data from 21 pure rectal adenomas (also indicated as A/A) from a previous study (12).

For intratumor heterogeneity analysis, three core biopsies were taken postoperatively, *ex vivo*, from 13 of the carcinoma cases and 5 of the pure adenoma cases at the surface of the tumor. These biopsies were randomly taken and snap frozen in liquid nitrogen. Biopsies contained either adenoma or carcinoma tissue.

The local medical ethical committee approved the study (protocol number P04.124). Table 1 shows all sample characteristics.

DNA isolation

Formalin-fixed, paraffin-embedded (FFPE) tissue from the adenoma and carcinoma fractions was analyzed. DNA was extracted as previously described (21). Briefly, three tissue punches (0.6 mm diameter) were obtained using a tissue microarrayer (Beecher Instruments, Sun Prairie, WI), and DNA was isolated with proteinase K. Formalin-fixed, paraffin-embedded DNA was subsequently cleaned up using the Genomic Wizard kit (Promega).

DNA from the frozen tumor biopsies was extracted as previously described (12) using the Genomic Wizard kit.

All DNA concentrations were measured with the PicoGreen method (Invitrogen-Molecular Probes, Breda, The Netherlands), and DNA quality was checked on a 1% agarose gel.

Array analysis

The use of SNP arrays is a well established method for copy number and LOH analysis. Therefore data were not validated with cytogenetics in the present study. Validation studies are well documented by us and others (22-24).

For each cell isolate, 1 μ g of DNA was used for the BeadArrays. Illumina BeadArrays, in combination with the linkage mapping panel version 4_v3 or version 4_v4B

(Illumina, San Diego, CA), were used and respectively contained 5,861 or 6,008 SNP markers distributed evenly over the genome with an average physical distance of 482 kb. Samples were prepared according to the Goldengate assay (25). Gene calls were extracted using the gene calling programs GeneCall and GTS Reports (Illumina, San Diego, CA).

Copy number and LOH analysis

Copy numbers were determined based on intensity of the individual SNPs (23). LOH was analyzed by comparing the genotypes from paired normal and tumor DNA. Analyses were done using the R-package beadarraySNP. In addition, chromosome visualization of LOH was done in Spotfire DecisionSite (Spotfire, Somerville, MA) (26). LOH was calculated as described². Briefly, LOH was computed from the gene call score and the gene train score output of GeneCall and GTS Reports (Illumina, San Diego, CA). LOH was called for high quality heterozygous SNPs in the normal tissue (gene call score/gene train score ratio > 0.8) that were, in the paired tumor, homozygous or showed a gene call score/gene train score ratio of <0.8. Only LOH at a stretch of two or more SNPs was scored (26). When both physical loss and LOH were detected at a specific region, the LOH detected is an additional indication of physical loss. In the case where no copy number change was detected, LOH was interpreted as copy neutral LOH.

APC and KRAS mutation screening

APC and *KRAS* mutation detection were performed as described (27)³. PCR product (5-10 ng) was sequenced with 6 pmol of M13 forward or reverse primer on an ABI 3700 DNA Analyzer using Big Dye Terminator Chemistry (Applied Biosystems, Foster City, CA). Sequences were analyzed with Mutation SurveyorTM DNA variant analysis software (version 2.61 Softgenetics, State College, PA).

p53 and SMAD4 immunohistochemical analysis

Triplicate tissue cores from tumor areas, selected by a pathologist (H.M.) based on (H&E)-stained slides, were taken from each specimen (Beecher Instruments, Silver Springs, MD, USA). These punches, which had a diameter of 0.6 mm, were arrayed on a recipient paraffin wax block using standard procedures (28, 29). A paraffin sectioning aid system (Instrumedics Inc., Hackensack, NJ) was used to facilitate cutting 5- μ m sections of the tissue micro-array. After antigen retrieval (microwave oven treatment for 10 minutes in 10

² R. van Eijk et al. Genotyping and LOH analysis on archival tissue using SNP arrays. In Genomics - Method Express, M.Starkey and R.Elaswarapu, eds. (Bloxham: Scion Publishing); 2008, in press.

³ M. van Puijenbroek et al. Identification of patients with (atypical) MUTYH-associated polyposis by KRAS2 c.34G > T prescreening followed by MUTYH hotspot analysis in formalin-fixed paraffin-embedded tissue. Clin Cancer Res. 2008 Jan 1;14(1):139-42.

mmol/L citrate buffer pH 6.0 (p53) or Tris-EDTA pH 8.0 (SMAD4)), endogenous peroxidases were inactivated by 1% H₂O₂/PBS. Sections were incubated overnight at room temperature with mouse anti-human monoclonal antibodies directed against p53 (clone D0-7, 1:1000 dilution; NeoMarkes) or SMAD4 (clone B-8, 1:100 dilution; Santa Cruz Biotechnology). The sections were then incubated and stained with a biotinylated secondary antibody in PBS/bovine serum albumin 1% (p53) or Envision HRP-ChemMate kit (SMAD4; DAKO). Diaminobenzidine tetrahydrochloride was used as a chromogen for p53 staining. The slides were counterstained with hematoxylin. p53 was scored in four different categories based on any level of nuclear staining: 1% to 25% positive nuclei (indicative for a wildtype status), 25% to 75% positive nuclei, > 75% positive nuclei (the latter two mostly indicative for a mutation) or completely negative (uninformative). SMAD4 was scored in the following categories: no nuclear staining with a positive internal control (total loss), weak nuclear staining (down regulation) and moderate to strong nuclear staining (positive).

Statistics

Student's *t*-test was used to compare means of continuous variables between two groups. χ^2 tests were done to test significance between groups for specific loss and gain events. Physical loss and copy neutral LOH were considered as identical events in these analyses. Correlations between two tumor fractions were computed using Pearson's correlation coefficients. For all analyses, *P*-values of <0.05 were considered as significant. All these analyses were done using Statistical Package for the Social Sciences 12 (SPSS).

Results

Chromosomal aberrations

In a previous study, we typed copy number profiles using SNP arrays in 77 fresh frozen tumors of different stages (12). We subdivided the adenoma tissue into pure adenomas (A/A) and adenoma fractions of cases with a carcinoma focus (A/C). The carcinoma tissue was subdivided in tumor samples consisting of a mixture of adenoma and carcinoma tissue (AC/C), carcinoma tissue alone (C/C) and primary tumors in cases with lymph node metastasis (C/C (N+)). Importantly, the latter two contained no or only minimal adenoma tissue, whereas the A/C cases consisted predominantly of adenoma tissue. We found five specific chromosomal aberrations (gain of 8q, 13q and 20q and loss of 17p and 18q), which could discriminate adenomas from carcinomas.

With the aim of studying the early aberrations already present in the adenoma fraction of carcinoma cases, we assessed copy number alterations and LOH in paired adenoma (A/C) and carcinoma (C/C) formalin-fixed, paraffin-embedded tissues of 36 TEM treated rectal carcinomas. In two cases, two different adenoma fractions were identified, and for

Table 1. Patient characteristics.

ID	Sex	Age	Size (cm)	Fraction analyzed	Carcinoma		Biopsy	Recurrence	Distant metastasis
					T-stage	N-stage			
1	M	63	8	LC	2			x	
2	F	70	3.5	LC	1			x	x
3	F	89		HC	1				
4	F	77	1.5	HC	1	1		x	
5	F	56	7.5	LC	3	1	aaa		
6	M	59	4.5	LC	2				
7	M	77	7	LC	2				
8	M	55	3	LH	1		aaa		
9	F	79		HC	2				
10	M	61	7	LC	1		aaa	x	x
11	M	74	2.5	HC	1				
12	M	60	5	HC	1				
13	F	56	3.8	HC	1				
14	F	77	4	LHC	2				
15	F	73	3	HC	1				
16	M	79	4	HC	1		ccc		
17	M	61	10	LH	1				
18	F	56	8.6	LH	1		aaa	x	
19	M	45	5	LC	1				
20	M	60	2.5	LC	1				
21	F	49	2	LC	1				
22	M	68		HC	2				
23	F	70	1.5	LC	1		aac		
24	F	58	1	LHC	1				
25	M	46	5	HC	2	1	acc		
26	M	53	3.5	HC	2		ccc		
27	F	47		HC	2				
28	F	83	9	HC	1			x	x
29	F	73	2	HC	1		aaa		
30	F	65		HC	1		aaa		
31	M	64	5	HC	2				
32	M	71	6.5	LC	1				
33	M	80	1	HC	1				
34	F	58	3	HC	1				
35	M	70	11	HC	1			x	
36	F	73	8	LH	1			x	
37*	M	82	13.5	L	0				
38	M	75	7.5	H	0				
39	F	72	5	H	0				
40	M	62	7.5	H	0				
41	M	75	8	H	0				
42	M	78	4	H	0				
43	F	87	2	L	0		aaa		
44	M	61	5	H	0				
45	F	87	5	H	0				
46	M	67	9	L	0				
47	F	74	2	L	0		aaa		
48	F	68	2	L	0				
49	F	52	6	L	0		aaa		
50	M	53	9	L	0		aaa		
51	F	52	6.5	L	0				
52	F	63	7.3	L	0				
53	M	60	6	L	0				
54	M	79	5	L	0		aaa		
55	M	73	6.5	H	0				
56	F	40	11	L	0				
57	F	81	4.5	H	0				
58	M	69	7.5		1		aaa		
59	M	73	3.5		1		aac		
60	F	83	2		2		ccc		

NOTE: x, a recurrence or distant metastasis. Abbreviations: L, adenoma with low-grade dysplasia; H, adenoma with high-grade dysplasia; C, carcinoma; a, adenoma tissue; c, carcinoma tissue.

* Case 37 to 57 are pure adenomas from the previous study.

Table 2. Common aberrations (%) in different tumor fractions.

	AA n=21	A/C L n=18	A/C H n=24	A/C n=42	C/C n=32	A/C H vs. L	p-value *		
							A/C vs. A/A	C/C vs. A/A	C/C vs. A/C
Adenoma events									
loss 1p36	19	39	29	33	38	n.s.	n.s.	n.s.	n.s.
loss 4q32-pter	29	11	13	12	22	n.s.	n.s.	n.s.	n.s.
LOH/loss 5q	29	50	38	43	38	n.s.	n.s.	n.s.	n.s.
gain 7p15-11	29	17	13	14	25	n.s.	n.s.	n.s.	n.s.
gain 12q13	19	22	8	14	12	n.s.	n.s.	n.s.	n.s.
Carcinoma events									
gain 8q22-24	10	17	21	19	41	n.s.	n.s.	0.01	0.042
gain 13q	5	17	33	26	59	n.s.	0.049	<0.001	0.005
loss 17p	14	28	33	31	44	n.s.	n.s.	0.02	n.s.
loss 18q12-22	14	33	46	40	66	n.s.	0.028	<0.001	0.031
gain 20q	10	33	46	40	47	n.s.	0.007	0.003	n.s.
gain 13q combined with loss 18q12-22	0	12	13	12	41	n.s.	0.037	<0.001	0.005
Lymph node metastasis									
gain 1q23	0	0	0	0	9	n.d.	n.d.	n.s.	0.023
Other progression events									
8p loss	5	6	8	7	34	n.s.	n.s.	0.007	0.003
14q loss	10	0	8	5	22	n.s.	n.s.	n.s.	0.024
15q loss	0	6	8	7	25	n.s.	n.s.	0.003	0.032
19q gain	5	0	4	2	16	n.s.	n.s.	n.s.	0.028
Mutations †									
KRAS	53 (9/17)	67 (10/15)	67 (12/18)	67 (22/33)	50 (12/24)	n.s.	n.s.	n.s.	n.s.
APC	61 (11/18)	76 (13/17)	50 (10/20)	62 (23/37)	46 (13/28)	n.s.	n.s.	n.s.	n.s.
KRAS & APC	28 (5/18)	47 (7/15)	32 (6/19)	38 (13/34)	16 (4/25)	n.s.	n.s.	n.s.	n.s.
Immunohistochemistry †									
P53	5 (1/20)	0 (0/13)	46 (10/22)	29 (10/35)	63 (17/27)	0.001	0.022	<0.001	0.006
SMAD4_faint ‡	48 (10/21)	88 (15/17)	64 (14/22)	74 (29/39)	81 (22/27)	n.s.	0.04	0.13	n.s.
SMAD4_neg ‡	0 (0/21)	18 (3/17)	18 (4/22)	18 (7/39)	41 (11/27)	n.s.	0.011	<0.001	n.s.

Abbreviations: n.s., not significant; n.d., not determined. *p-values were computed by χ^2 test. † For both mutational analysis and immunohistochemistry, not all cases could be typed, due to technical limitations. For each group the number of typed individuals with a mutation/staining and the total number typed are indicated in brackets. ‡ Reduced expression of SMAD4 protein expression (SMAD4_faint); completely negative for SMAD4 protein expression (SMAD4_neg).

four cases, the carcinoma fraction was too small to be analyzed; therefore, both the adenoma fraction with low and high grade dysplasia were analyzed, finally leading to a total number of 32 C/C fractions and 42 A/C fractions. Table 2 shows the most frequent chromosomal changes per sample group; in supplementary Table 1, all genomic and genetic abnormalities are shown for each case. The A/C and C/C fractions were compared with each other and with the pure adenomas (A/A) from the previous study (12). From that study, we learned that only specific adenoma events (loss of 1p36, 4q32-pter and 5q and gain of 7p15-11 and 12q13) were frequently involved in the A/A cases. In the current study, we observed that the carcinoma or “malignant” events were all significantly different between the C/C and A/A groups (Table 2). Three of the five events were also significantly different between the A/C and A/A groups (13q gain, 20q gain, and 18q12-22 loss) and between the C/C and A/C groups (8q22-24 gain, 13q gain, and 18q12-22 loss). In addition,

13q gain combined with 18q loss was significantly different between the groups. Moreover, additional carcinoma progression events were identified in this study: loss of 8p, 14q and 15q and gain of 19q were all increased in carcinoma fractions (C/C) in comparison with their adenoma counterparts (A/C).

Mutations of *APC* and *KRAS*

To supplement chromosomal instability data, mutational status of colorectal cancer genes *APC* and *KRAS* was studied. A major function of the APC protein is β -catenin degradation. Mutations in *APC* result in the loss of β -catenin binding sites; however, when the mutation is in the mutation cluster region, one or two active β -catenin binding sites are retained. Albuquerque et al. (30) posed that the position and type of the second hit on *APC* depends on the localization of the first hit. Patients with the first mutation around codon 1300 acquire the second hit by allelic loss, whereas patients with a first mutation elsewhere acquire truncating mutations within the mutation cluster region rather than loss/LOH. The amount of remaining β -catenin binding sites might lead to a different biological behavior of the tumors. For *KRAS*, the type of mutation was also suggested to be of significance. In a large data set, the valine alteration was correlated with shorter survival in relation to other mutations (31).

In the pure adenomas, we observed high percentages of *APC* and *KRAS* mutations (61% and 53% respectively), comparable with frequencies in the A/C (62% and 67%) and C/C tumor fractions (50% and 46%, Table 2). For *APC*, we examined whether patients with 5q retention had other types of *APC* mutations compared with cases with 5q LOH/loss. In the cases with 5q LOH/loss, we observed that 64% had an *APC* mutation, whereas cases with 5q retention showed a frequency of 52% (not significant). There was no difference in the type of mutation and, consequently, in the amount of remaining β -catenin binding sites, among A/A, A/C and C/C samples. For *KRAS*, we examined if we could detect any difference in type of mutation. Glycine to valine and glycine to aspartic acid were the most frequent alterations (n=11 and n=13, respectively). However, no difference in type of mutation was observed among A/A, A/C, and C/C samples. The A/C group had the most double mutations; 38% had a mutation in both *APC* and *KRAS*, compared with 28% for the A/A cases and 16% for the C/C cases. However, this difference was not significant.

p53 and SMAD4 immunohistochemistry

Not many target genes on chromosomes 8q, 13q, 17p, 18q and 20q have been unequivocally identified. However, the role of *p53* on 17p and *SMAD4* on 18q has been amply documented in the tumorigenesis of CRC (32-35). Nevertheless we cannot rule out completely that other genes are targeted by these chromosomal aberrations as well. Because reliable immunohistochemistry was available, we did p53 and SMAD4

immunohistochemical staining on tissue microarrays and correlated the findings to allelic loss status. Although 17p loss frequency was only significantly different between the A/A and C/C tumors, aberrant p53 staining (25-100% positive nuclei, indicative for a mutation) was significantly increased in the A/C group compared with the A/A group (29% versus 5%, $P = 0.022$) and in the C/C group compared with both the A/C and A/A groups (63% versus 29%, $P = 0.006$ and 63% versus 5%, $P < 0.001$, respectively; Table 2). In addition, significantly more aberrant staining was observed in the A/C group with high-grade dysplasia compared with low-grade dysplasia (46% versus 0%, $P < 0.001$). For SMAD4, we assessed both the percentage of down-regulation and complete loss of protein expression. Down-regulation of SMAD4, as well as complete loss of SMAD4, were both significantly increased in the A/C cases when compared with the A/A cases (74% versus 48%, $P = 0.04$ and 18% versus 0%, $P = 0.011$, respectively), and complete loss of SMAD4 was different between C/C and A/A cases (41% versus 0%, $P < 0.001$; Table 2). Both down-regulation

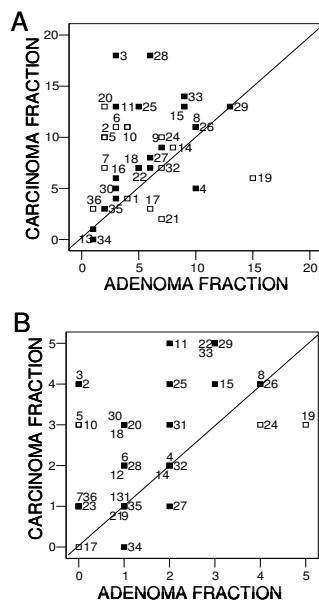


Figure 1. A and B, all 36 adenoma-carcinoma pairs are plotted against each other. X axis, adenoma fraction; Y axis, matching carcinoma fraction. Respectively, the amount of all aberrations (A) and the five malignant events (B) are shown. A, the degree of dysplasia for the adenoma fraction is indicated (white, low-grade dysplasia; black, high-grade dysplasia). Numbers in the plot indicate the sample ID. B, several pairs coincide in the same data point. For cases 8, 17, 18, and 36, no carcinoma fraction was analyzed (see Table 1), and for these samples, we compared the adenoma with low- versus high-grade dysplasia. For samples 14 and 24, the adenoma fraction with low-grade dysplasia was plotted.

and complete loss of SMAD4 expression were correlated with 18q loss ($P = 0.018$, $P = 0.011$, respectively).

Association of chromosomal aberrations to clinicopathologic features

We investigated whether several clinicopathologic markers were associated with chromosomal aberrations. The malignant tumors were significantly smaller than the pure adenomas (mean diameter 4.6 versus 6.3 cm, $P=0.032$); however, the total number of aberrations, or the amount of the five malignant aberrations, did not correlate with tumor size. Furthermore, samples from different T stages were compared. We compared 10 T2 carcinomas with 25 T1 carcinomas. No significant differences were observed between these groups in total chromosomal instability or malignant aberrations. Nine cases with local recurrences were compared with those without recurrences. However, no significant differences were observed. Three samples had lymph node metastasis, but this number was too small to make any comparisons.

Systematic comparison of adenoma and carcinoma tissue in the same lesion

Figure 1 shows a systematic comparison between the adenoma and carcinoma fraction of single cases for all genomic aberrations. Most data points are slightly above the $x=y$ line, indicating that carcinoma fractions have slightly more aberrations than the corresponding adenoma fractions. Correlation coefficients between adenoma and carcinoma fractions were 0.229 ($P =0.180$) and 0.516 ($P =0.001$) for the total number of aberrations and the five “malignant” aberrations, respectively. The adenoma fractions with low-grade dysplasia showed fewer aberrations than the adenoma fractions with high grade dysplasia; however, this difference was not significant. Four carcinoma fractions (11%) showed the same number of aberrations as their corresponding adenoma fraction, whereas 47% showed one to five extra events, and 28% showed more than five extra events in the carcinoma fraction (Figure 1A). For five cases (14%), the adenoma fraction contained more aberrations than its corresponding carcinoma fraction.

Figure 1B compares the occurrence of the five malignant aberrations between the adenoma and carcinoma fraction in the same lesion. In 42% of the adenoma fractions, two or more malignant events were identified. In 11 cases (31%), the amount of malignant events was identical in the adenoma and carcinoma fraction of one tumor. In 25% of all cases, one extra malignant event was detected in the carcinoma fraction, whereas in 33% two or more extra malignant events were detected. In four cases (11%), the adenoma fraction contained more malignant aberrations than the carcinoma fraction. For cases with more aberrations in the adenoma than in their carcinoma counterparts, we determined if data were in accordance with immunohistochemistry. For instance, in case 24, the adenoma fraction showed loss of 18q and reduced SMAD4 protein expression, whereas the

carcinoma fraction showed 18q retention and a normal SMAD4 staining pattern. In the other samples, immunohistochemistry also confirmed chromosomal aberrations.

Table 3 shows the distribution of the genomic changes over the chromosomes. As expected, the malignant aberrations were the most common “progression” events, as these had the highest frequency in the carcinoma fractions, while the corresponding adenoma fractions did not show this event. 13q and 18q were especially strongly increased; in 13 and 14 cases, respectively, the carcinoma fraction contained this event in contrast to the adenoma fraction. The other extra events in the carcinoma fractions did not involve specific chromosomes, as the random distribution of events over the chromosomes shows.

Intratumor heterogeneity analysis in tissue biopsies

For the clinical application of chromosomal instability profiling, accurate analysis of preoperative tissue biopsies is essential. To mimic these biopsies, we investigated three

Table 3. Distribution of genomic alterations over the chromosomes in adenoma and carcinoma fractions of single lesions (n=36)

Chromosome	Aberrations present in both fractions	Aberrations present in carcinoma fraction, not in adenoma fraction	Aberrations present in adenoma fraction, not in carcinoma fraction
1p	10	6	5
1q	4	0	1
2p	1	1	0
2q	2	1	0
3p	1	3	2
3q	0	1	0
4p	3	4	3
4q	4	5	1
5p	0	4	2
5q	12	4	4
6p	5	7	2
6q	3	4	1
7p	8	3	0
7q	7	4	1
8p	4	9	2
8q	5	8	2
9p	2	4	1
9q	3	3	2
10p	1	5	1
10q	2	5	2
11p	0	2	0
11q	1	2	1
12p	7	4	2
12q	7	4	1
13q	9	13	1
14q	2	6	1
15q	3	7	1
16p	2	4	1
16q	3	3	2
17p	10	5	3
17q	5	0	5
18p	10	10	2
18q	11	14	2
19p	3	3	0
19q	1	5	0
20p	7	7	2
20q	9	9	2
21q	4	4	1
22q	6	3	2

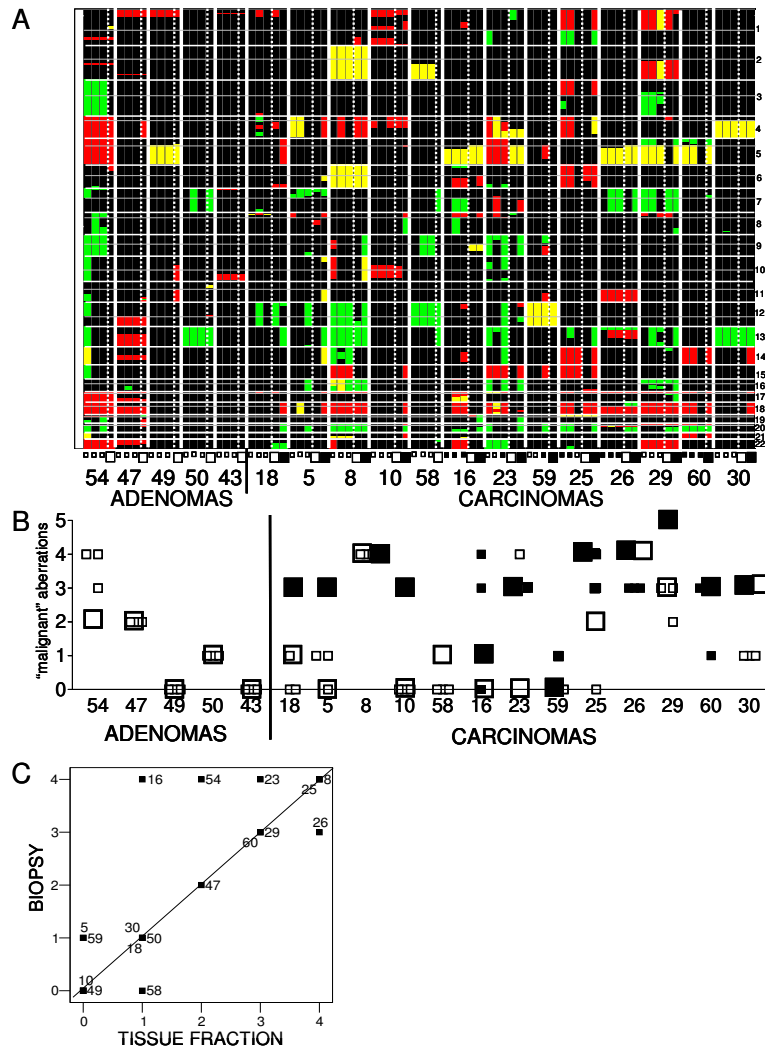


Figure 2. A to C, overview of the 5 pure adenomas and the 13 adenoma carcinoma pairs from which three biopsies per tumor were analyzed. We show the three biopsies per tumor, the adenoma fraction, and the carcinoma fraction, respectively. Numbers on the X axis indicate the sample ID, whole tumor fractions are indicated by large squares, and biopsies are indicated by small squares (*white*, adenoma tissue; *black*, carcinoma tissue). A, all different aberrations are shown for every sample and all chromosomes. Green, gain; red, loss; yellow, copy number neutral LOH. B, amount of five malignant aberrations per tissue sample. C, the amount of the five malignant aberrations for the whole tissue fraction (X axis) against the biopsy with the most aberrations (Y axis) was plotted per tumor sample. Labels in the plot indicate the sample ID.

postoperative biopsies for each tumor and estimated how representative these biopsies are for the tissue sample because intratumor heterogeneity is a well-known phenomenon in colorectal cancer.

Three different biopsies were postoperatively taken *ex vivo* from five pure adenomas and 13 carcinomas at random positions from the surface of the tumor and analyzed with SNP arrays (Table 1). Figure 2A shows genome wide chromosomal aberrations in the different biopsies and their corresponding adenoma or carcinoma fraction. Roughly the same pattern of aberrations is seen in the different biopsies and the corresponding tumor fraction of the same patient. The number of “malignant” aberrations for all three biopsies, and the adenoma and carcinoma fractions per patient, is comparable for most cases (Figure 2B). In 3 out of 18 (17%) tumors (cases 16, 30 and 54), the amount of “malignant” aberrations differed considerably between the biopsies and the tumor fractions, whereas in the majority of cases (15 out of 18, 83%) the biopsies showed one different “malignant” aberration at most.

We hypothesized that the biopsy with the largest number of chromosomal aberrations is representative for the tumor. Correlation coefficients for the number of total aberrations and for the number of “malignant” aberrations between that biopsy and the corresponding tissue fraction were 0.660 ($P = 0.003$) and 0.807 ($P < 0.001$; Figure 2C), respectively (biopsies containing adenoma tissue were compared with adenoma fractions, and carcinoma biopsies were compared with carcinoma fractions). We simulated the effect of taking, at random, one or two biopsies (instead of three). Taking only one biopsy resulted in a lower correlation, whereas the effect of two biopsies was nearly comparable with that of three biopsies (data not shown).

Discussion

For correct preoperative staging of rectal tumors, especially large sessile adenomas eligible for TEM resection, it is necessary to identify those adenomas already containing an invasive focus. In a previous study, we found that five specific chromosomal aberrations could clearly discriminate sessile adenomas from carcinomas (12). Moreover, in adenoma fractions from cases with a carcinoma, twice the amount of such “malignant” aberrations was observed, as compared with pure adenomas. In the present study, we analyzed the adenoma and carcinoma fractions of 36 rectal tumors and found that two or more malignant events are present in 46% of the adenoma fractions and that the increase in malignant aberrations in adenoma to carcinoma progression was relatively small. Intratumorheterogeneity analysis showed that it is essential to analyze multiple biopsies for a correct assessment of chromosomal instability patterns.

The Vogelstein progression model for colorectal tumorigenesis, proposed in 1990 and adapted in the years after, has been addressed by many other studies (20, 36-38). We now seek to use such data for clinical decision making. Our study showed that three of the five malignant events (gain of 13q and 20q and loss of 18q) were already abundant and significantly increased in rectal adenoma fractions of carcinoma cases compared with pure adenomas. The two other malignant events (8q gain and 17p loss) were not significantly changed, but percentages were increased. Furthermore, 17p loss was related to aberrant nuclear staining for p53 using immunohistochemistry, which was significantly different in adenomas with a carcinoma focus versus pure adenomas. Loss of 18q and SMAD4 immunohistochemistry showed an identical relationship. The relative additional amount of chromosomal aberrations in the transition from adenoma to carcinoma was most often equal in cases with a limited amount of adenomatous aberrations to those with a high amount of such events.

Hermesen et al. (15), described seven cancer-associated events (loss of 8p, 15q, 17p and 18q and gain of 8q, 13q and 20q) that were associated with both carcinomas and adenoma fractions of carcinomas. In addition, they found that these chromosomal abnormalities occurred in specific combinations of a few abnormalities rather than as a mere accumulation of events. We did not identify a specific combination of events but found that most carcinomas have at least two of the five malignant events. In addition, we identified gain of 19q and loss of 8p, 14q, and 15q as later events in carcinoma progression, as these were increased in the carcinoma fractions (C/C) compared with the adenoma fractions (A/C). These regions are, in part, similar to the results of Diep et al., who reported deletion of 8p and 14q and gain of 1q and 19q as late events that correlated with metastasis in a meta-analysis of 859 colorectal cancers (11).

SNP array analysis of three different *ex vivo* core biopsies per tumor showed a large degree of intratumor heterogeneity. Hence, it is essential to analyze several tumor fractions per patient for an accurate assessment of genetic changes. Although intratumor heterogeneity is a well-studied phenomenon in CRC (19, 20, 39, 40), our study is the first to assess genome wide heterogeneity through SNP array analysis in a series of rectal tumors. Losi et al. (20) found intratumor heterogeneity in 90% of early colorectal cancers, a percentage that corresponds to our data. In addition, Baisse (40) found heterogeneity in 67% of colorectal cancer. Studies in colorectal and other cancers showed that accumulation of clonal diversity is a fundamental principle in cancer progression (41-43). In our study, less heterogeneity was present when only the five malignant aberrations were tested. Moreover, a good correlation was established between the biopsy with the most aberrations per patient and the corresponding adenoma or carcinoma fraction. In spite of the observed heterogeneity, it seems that three biopsies per tumor can reliably assess the chromosomal aberrations in rectal tumors.

Surprisingly, some adenoma fractions showed more aberrations than their carcinoma counterparts. Likewise, several biopsies contained other or more aberrations than their corresponding tumor fraction. This interesting finding can be explained by different factors. First, tumor heterogeneity might be a reason; the carcinoma fraction of such a case might have arisen from a different tumor clone than the adenoma fraction studied. The fact that four cases showed either APC or KRAS mutations in the adenoma fraction and not in the carcinoma fraction also suggests that the carcinoma did not arise from the adenoma clone. Consistent with our findings, Zauber et al. (44) found a difference between the adenoma and carcinoma portion of tumors with regard to the KRAS gene in 24% of 37 neoplasms. Second, it was frequently observed that a carcinoma fraction had a larger stromal involvement and thus a somewhat lower tumor cell percentage than the adenoma fraction. Although a lower tumor cell percentage might make it more difficult to depict chromosomal aberrations, most aberrations seemed very reproducible. However, with too many contaminating stromal cells, a certain chromosomal aberration might be present in too few cells to be detected by current techniques. Laser capture microdissection might offer a solution for research, but is not feasible for a clinical application.

A recent study showed that retention of chromosome 5q correlated with liver metastasis in colorectal cancer (45). The authors found that tumors with 5q deletion (loss or LOH) have a different type of APC mutation than cases with 5q retention. Cases with 5q deletion usually have one APC allele affected by a mutation, usually leaving one or two β -catenin binding sites, whereas cases with retention usually have two different APC mutations (30). This can lead to differences in residual β -catenin activity, which in turn can show an effect on the neoplastic process. We did not detect any significant difference between cases with 5q retention versus 5q LOH/loss regarding APC mutations in the mutation cluster region. However, we analyzed only the mutation cluster region of APC, starting at codon 1284. If mutations occurred before the mutation cluster region, this would lead to loss of all β -catenin binding sites in one allele, as is probably the case in the samples with 5q retention.

As a large proportion of presumed sessile rectal adenomas seem to identify postoperatively as carcinomas, there is a need for additional preoperative tests. Most carcinomas in this study were preoperatively classified as adenomas; thus, a TEM was done. In the majority of carcinoma cases, preoperative and *ex vivo* core biopsies contained adenoma tissue, indicating that it is difficult to obtain a correct preoperative diagnosis using standard histopathology. Interestingly, 15 out of 36 (42 %) adenoma fractions of carcinoma cases had two or more malignant aberrations, indicative of malignancy. Aberrant p53 and SMAD4 immunohistochemical staining correlated with 17p and 18q loss, respectively, and were both increased in adenoma fractions of carcinomas in contrast to pure adenomas. Such p53 immunohistochemistry showed an even better discrimination between pure adenomas

and adenoma fractions of carcinoma cases than 17p loss, indicating that some cases might have two somatic mutations in the *p53* gene, instead of one mutation combined with chromosomal loss. However, we cannot exclude that other genes might be targeted by the loss. For chromosome 8q, 13q and 20q gain, the target genes are largely unknown, although a prime target on 8q might be the *cMyc* gene, for example. *BRCA2*, *Rb* and other tumor suppressor genes locate on chromosome 13q. Although 13q loss is observed in most cancer types, this chromosome usually shows gain in colorectal cancer. Earlier observations indeed showed increased copy numbers of one *Rb1* allele, and increased levels of *Rb* mRNA and protein expression in CRC (46-48). The role of *Rb* in colorectal cancer development is thus not clear. Currently we are integrating gene expression analysis with the obtained SNP data in order to study the effect of chromosomal aberrations on the transcriptional level.

Our *ex vivo* biopsy analysis showed that the analysis of small biopsies is feasible because the chromosomal aberrations were reliably identified. Additionally, biopsies were taken at the surface of the tumor, just as in the preoperative situation. The five chromosomal regions and immunohistochemistry for p53 and SMAD4 should now be evaluated on a large series of multiple preoperative biopsies. However, reservations may exist to the application of the above approach, given that some adenomas tend to harbor more aberrations than their carcinoma counterparts. After validation studies, these methods can hopefully be added to future histological analysis and imaging methods, possibly leading to improved rectal tumor staging.

In conclusion, adenoma fractions of rectal carcinoma cases show a high degree of chromosomal instability and have a relatively small increase in genomic alterations in their transition to carcinomas. The occurrence of specific chromosomal events could possibly be used to predict the malignant behavior of sessile rectal adenomas. The analysis of several biopsies per patient revealed a large degree of intra-tumor heterogeneity, but when three biopsies per tumor are analyzed, most aberrations are reliably identified.

Acknowledgments

We thank Stichting Laboratorium Pathologie en Cytologie (Rotterdam, the Netherlands) and Stichting Samenwerking Delftse Ziekenhuizen (Delft, the Netherlands) for providing the tissue samples and Hans Halfwerk for technical assistance. This project was supported by Dutch Cancer Society grant RUL 2003-2807.

Supplementary data

Supplementary data are available at
<http://clincancerres.aacrjournals.org/cgi/content/full/14/3/772/DC1>

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