

Colorectal adenoma to carcinoma
progression follows multiple pathways
of chromosomal instability

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CHAPTER 2

Abstract

Background & Aims: Current models of colorectal adenoma to carcinoma progression do not fully reflect the genetic heterogeneity and complexity of the disease. The aim of the present study was to identify genetic changes discriminating adenomas that have progressed to carcinoma from adenomas that have not progressed, and to refine the current genetic models of colorectal adenoma to carcinoma progression, based on a genome-wide analysis of chromosomal aberrations.

Methods: Sixty-six nonprogressed colorectal adenomas, 46 progressed adenomas (malignant polyps), and 36 colorectal carcinomas were screened for chromosomal aberrations by comparative genomic hybridization, and for mutations in the *adenomatous polyposis coli* (*APC*) and *K-ras* gene. Data analysis focused on cancer-associated genetic changes in adenomas.

Results: Accumulation of losses in 8p21-pter, 15q11-q21, 17p12-13, and 18q12-21, and gains in 8q23-qter, 13q14-31, and 20q13 were strongly associated with adenoma-to-carcinoma progression, independent of the degree of dysplasia. Hierarchic cluster analysis demonstrated the presence of 3 distinct subgroups of adenomas, characterized by unique combinations of genetic aberrations in the adenomas (17p loss and *K-ras* mutation, 8q and 13q gain, and 18q loss and 20q gain, respectively).

Conclusions: The presence of 2 or more of the aforementioned 7 chromosomal changes was associated with progressed colorectal adenomas and colorectal cancer. In addition, evidence was found that these chromosomal abnormalities occurred in specific combinations of a few abnormalities rather than as a mere accumulation of events, indicating the existence of multiple independent chromosomal instability pathways of colorectal cancer progression.

Introduction

Colorectal adenomas are highly prevalent lesions occurring in more than 30% of the normal population over age 60 years [1]. However, only about 5% of these adenomas ever progress to cancer. The ability to distinguish adenomas that do progress to cancer from those that will not progress is highly relevant for colorectal cancer screening. Because this difference cannot be reliably made macroscopically, endoscopic screening strategies aiming to detect and remove all adenomas will be inherently unspecific – most of the adenomas removed would not have progressed to cancer anyway. Because colorectal tumor progression is driven by an accumulation of genetic abnormalities, genetic testing focused specifically at these genetic alterations may show a higher specificity for discriminating adenomas with a high risk of progression from adenomas that will not progress [2]. Theoretically, achieving this goal would require a prospective study design in which DNA is sampled from colorectal adenomas, which are subsequently left in situ for 10-15 years to see whether or not they progress to cancer. However, this is not feasible. Instead, cross-sectional studies must be used to obtain this information.

Therefore, in this article we present a cross-sectional study of a total of 194 colorectal tumor samples, comparing genetic data from colorectal adenomas that had demonstrated a capacity for progression to carcinoma (because they already contained a focus of carcinoma) to a random set of nonprogressed adenomas without evidence of cancer at the time of resection. Because in general, only 5% of colorectal adenomas will ever progress to cancer, this can be regarded as a reliable set of controls. In addition, a set of colorectal carcinomas was included. Chromosomal aberrations were analyzed on a genomewide scale, using comparative genomic hybridization, supplemented by mutation analysis of *APC* and *KRAS*. Data analysis focused on cancer-associated genetic changes in adenomas. Using hierarchic cluster analysis, we set out to define subsets of tumors with distinct patterns of these cancer-associated chromosomal aberrations.

Concerning the molecular pathways underlying tumor progression, we know that genetic changes lead to the disruption of critical biologic functions and that they arise as a consequence of genomic instability, which in sporadic colorectal cancer occurs mainly at the chromosomal level [3]. Our current understanding of colorectal tumorigenesis has been based on the analysis of a relatively limited number of genetic changes [4-6], and thus has resulted in models suggesting a linear sequence of events leading to cancer. In the model presented by Vogelstein et al. [7], *KRAS* mutation and loss of heterozygosity on chromosome 5q (the *APC* tumor-suppressor gene locus) are early genetic changes, whereas loss of heterozygosity of 18q (initially *DCC*, but later *Smad4*) and 17p (*p53*) were considered late events.

Although this model has been revolutionary in our understanding of the multistep pathogenesis of human sporadic colorectal cancer, it now appears that it cannot fully explain the heterogeneity and complexity of colorectal cancer. Several frequent chromosomal aberrations are not taken into account, including losses of 8p and 15q and especially gains of 8q, 13q, and 20q, as has been revealed by genome-wide chromosomal analysis using comparative genomic hybridization (CGH) [8-11]. How all of these events would fit into a linear model of tumor progression is not immediately clear. Only the study of large numbers of cases based on the analysis of many genetic parameters simultaneously will allow the unravelling of the complex relationships between these events, enabling more exact modeling.

Material and Methods

Tumor material

A total of 194 tumor samples were studied, consisting of 66 nonprogressed adenomas (i.e., simple adenomas without invasion), 46 progressed adenomas (in which a focus of carcinoma already is present, also referred to as malignant polyps), of which both the adenoma part and the carcinoma part were analyzed separately; and 36 simple carcinomas. The samples were obtained from 93 patients, 41 female and 52 male. The mean age was 67 years (range, 40-91). Thirty patients had multiple tumors: 5 had multiple adenomas, and 25 had 1 or more adenomas next to a carcinoma. Histopathologic characteristics are listed in Table 1.

Analysis of grade of dysplasia and histologic type of adenoma, as well as differentiation grade and stage of carcinoma, was performed by 1 observer (G. M.) on hematoxylin and eosin-stained sections. For each tumor tissue sample, DNA was extracted from 15 10- μ m paraffin sections, dissecting the most tumor-rich areas, allowing a maximum of 20% nontumor cell contamination [12]. Special care was taken to obtain highquality DNA from the formaldehyde-fixed, paraffin-embedded tissues. DNA extracted from archival material can be partly degraded and cross-linked, the extent of which depends on the pH of the formaldehyde and the time of the fixation before paraffin embedding. To improve the quality of the isolated DNA, we have developed a very elaborate DNA extraction protocol especially for paraffin tissues, based on the protocol published by Isola et al. [13]. It includes thorough deparaffination with xylene, methanol washings to remove all traces of the xylene, and a 24-hour incubation in 1 mol/L sodium thiocyanate to reduce cross-links. Subsequently, the tissue pellet is dried and digested for 3 days in lysis buffer (155 mmol NH₄Cl, 10 mmol KHCO₃, 0.1 mmol Na₂EDTA, pH 7.4) with high doses of proteinase K (final concentration 2 μ g/ μ L, freshly added twice a day). Finally, the DNA is purified with Qiagen columns. With this

protocol, described in detail by Weiss et al. [14], generally around 90% of all formaldehyde-fixed, paraffin-embedded tissue samples yield DNA of good quality and lengths between 2000 and 20,000 bp. The present study included only cases that yielded good-quality DNA

Table 1. Histologic characteristics of 194 colorectal adenoma and carcinoma samples

	Adenomas	n	Carcinomas	n
Samples	Non-progressed adenomas: nA	66	Carcinomas, part of malignant polyps: C(mp)	46
	Progressed adenomas, part of malignant polyps: pA	46	Simple carcinomas: C(s)	36
Histologic type	Tubular	51		
	Tubulovillous	50		
	Villous	9		
	Serrated	2		
Dysplasia	Mild	20		
	Moderate	61		
	Severe	31		
Differentiation grade			Well	15
			Moderate	62
			Poor	5
Astler-Coller Stage			A	26
			B1	18
			B2	15
			C1	4
			C2	11
			D	2

The DNA was used for CGH analysis and mutation analysis of *KRAS* and *APC*. CGH was completed in all cases, but for the other tests it was not possible to perform all analyses on all tumor samples. Therefore, the number of cases analyzed is shown between brackets for each analysis.

Comparative Genomic Hybridization

CGH (n=194) was performed as previously described [14]. Normal metaphase spreads were obtained by culturing lymphocytes from a healthy female donor, according to standard procedures. Normal reference DNA was obtained from peripheral blood lymphocytes of a healthy female, and tumor DNA from 20 10- μ m sections of paraffin tissue using the Qiagen DNA isolation kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's recommendations. Tumor DNA was labeled with biotin-16-dUTP (Roche Diagnostics, Almere, The Netherlands) and the reference DNA with digoxigenin-11-dUTP (Roche Diagnostics) by nick translation. The concentration of DNase I (Gibco BRL, Breda, The Netherlands) was adjusted so that the length of the labeled DNA fragments was 0.5-1.5 kilobase. Then 500 ng of biotinlabeled tumor and 500 ng of digoxigenin-labeled normal DNA, together with 40 μ g unlabeled Cot-1 DNA (Gibco BRL) were precipitated with ethanol, and the pellet was dissolved in 6 μ L 50% formamide, 10% dextrane sulphate in 2x saline sodium citrate (SSC; 0.3 mol/L sodium chloride, 0.03 mol/L sodium citrate, pH 7.0). This probe mixture was denatured at 80°C for 10 minutes. Normal metaphase slides were denatured at 72°C for 6 minutes in a coplin jar containing 70% formamide in 2x SSC, pH 7.0. The probe mixture was mounted onto the slides, sealed with a cover slip, and hybridized for 3 days in a humid incubator at 40°C. The slides were washed for 5 minutes in 2x SSC at room temperature, 3 times for 5 minutes in 0.1x SSC at 45°C, and 5 minutes in 0.05% Tween-20 in TN (100 mmol Tris-HCl, pH 7.6, 150 mmol sodium chloride). The slides were then preincubated for 10 minutes at 40°C in blocking solution (0.5% blocking reagent; Roche Diagnostics, Almere, The Netherlands) in TN, followed by a 60-minute incubation at 40°C with 12.5 μ g/mL fluorescein isothiocyanate (FITC)-conjugated avidin (Sigma-Aldrich, Poole, Dorset, England) and 4 μ g/mL tetramethylrhodamine isothiocyanate (TRITC)-conjugated sheep antidigoxigenin (Roche Diagnostics) in blocking solution. The slides were washed 3 times for 5 minutes at room temperature in 0.05% Tween-20 in TN and dried in an ethanol series. Finally, the slides were mounted with 20 μ L antifade solution (Vectashield; Vector Laboratories, Burlingame, CA) containing 0.35 μ g/ μ L 4, 6-diamidino-2-phenylindole. The slides were analyzed on a Leica DM-RA microscope equipped with filter sets for 4,6-diamidino-2-phenylindole, FITC, and TRITC. Image acquisition and analysis was performed with the Cytovision CGH software package 3.5 (Applied Imaging, Sunderland, England). The average green-to red fluorescence ratios of chromosomes of 10-15 metaphases was plotted along ideograms of the corresponding chromosomes, together with the 95% confidence interval. 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. Chromosomes 1p32-pter, 16, 19, and 22 were excluded from further analyses, because these regions may yield unreliable CGH data caused by repetitive DNA sequences.

APC mutation analysis

The procedure for amplification of the mutation cluster region of the *APC* gene (n=135) as 4 overlapping fragments entailed a nested polymerase chain reaction (PCR) strategy [15]. Outside PCR was carried out to generate 2 fragments, A (356 bp, Nt position 3874-4229) and B (511 bp, Nt position 4114-4624). Genomic DNA (50 ng) was added to 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 2.0 mmol MgCl₂, 250 μmol/L dNTP (Pharmacia, Uppsala, Sweden), 200 nmol/L of each primer (fragment A: sense, GAAATAGGATGTAATCAGACG, antisense, AGCTGGCAATCGAACGACT; fragment B: sense, GCTCAGACACCCAAAAGTCC, anti-sense, CATTCCCATTGTCAATTTCC), and 1 U Platinum-Taq (Gibco BRL) in a final volume of 50 μL. Fragment A was used as starting material for the amplification of nested fragments S1 (219 bp) and S2 (204 bp), and fragment B was used for nested fragments S3 (205bp) and S4 (267 bp). The inside PCR was performed in a final volume of 50 μL, containing 5 μL of a 1:100 dilution of the outside product, 50 mmol/L KCl, 10 mmol Tris-HCl (pH 8.3), 1.5-2.5 mmol/L MgCl₂, 250 μmol/L dNTP, 200 nmol/L of each primer (fragment S1: sense, biotin-AAATAGGATGTAATCAGACG, antisense CGCTCCTGAAGAAAATTCAAC; fragment S2: sense, biotin-ACTGCAGGGTTCTAGTTTATC, antisense, GAGCTGGCAATCGAACGACT; fragment S3: sense, biotin-TACTTCTGTCAGTTCACCTTGATA, antisense, ATTTTTAGG-TACTTCTCGCTT; fragment S4: sense, AAACACCTCCACCACCTCC, antisense, biotin-GCATTATTCTTAATTCCACATC), and 1 U Platinum Taq. The 4 inside fragments were labeled with biotin at 1 of the 2 primers for subsequent direct sequencing. The cycles for both outside and inside PCR were 3 minutes of pre-denaturation at 94°C; 40 seconds of denaturation at 94°C; 1 minute at 52°C (fragments S1 and S2), 54°C (fragments A and B), or 56°C (fragments S3 and S4) for annealing; 1 minute at 72°C for elongation; and 10 minutes at 72°C for postelongation. In each PCR, 1 round of 35 cycles was carried out using a Peltier Thermal Cycler 200 (Biozym Diagnostik GmbH, Oldendorf, Germany) or an iCycler (BioRad Laboratories B.V., Veenendaal, The Netherlands). In each PCR, positive (DNA from Caco2 cells) and negative controls (no DNA) were included. The length and concentration of the PCR products were checked by electrophoresis on 2% agarose gels and visualized with ethidium bromide.

The sequencing reactions for the biotinylated products of the inside PCR were carried out using an autoloading solid-phase sequencing kit (Amersham Biosciences Europe GmbH, Roosendaal, The Netherlands) with extended Cy5-labeled primers according to the manufacturer's instructions. Briefly, the PCR product is captured on a sequencing comb coated with streptavidin. After removal of the nonbiotinylated strands by alkaline denaturation, the remaining immobilized strand is used as a template for dideoxy sequencing reactions with a Cy5-labeled primer and T7 DNA polymerase. In this system, only full-length immobilized PCR product is available for sequencing. The purified

fragments were sequenced on an ALF Express (Amersham Biosciences Europe GmbH) DNA sequencer, and the results were analyzed using the ALFwin sequence analyzer 2.10 program (Amersham Biosciences Europe GmbH).

KRAS mutation analysis

KRAS mutation analysis (n=78) was done by PCR using an oligonucleotide 20-mer panel of codons 12 and 13 (TIB Molbiol; Advanced Biotechnology Center, Genova, Italy) as previously described [16]. Extracted DNA from peripheral blood lymphocytes from healthy donors was used as wild-type KRAS codon 12 GGT-gly and codon 13 GGC-gly controls, and extracted DNA from 6 different colon cancer cell lines was used as control for known KRAS mutations.

Statistical analyses

The Student *t* test and 1-way analysis of variance were used for comparing means of continuous variables between 2 or more groups. For testing significance of differences in distribution of categorical variables, cross-tables were analyzed with the 2-sided Fisher exact test and Pearson's test, depending on the number of categories. *P* values < 0.05 were considered significant.

To analyze the presence of subsets of adenomas and carcinomas with comparable patterns of chromosomal aberrations, as well as analyze correlations between specific chromosomal aberrations, 2-dimensional hierarchical cluster analysis of the CGH data was performed. Cluster and Treeview software (<http://rana.lbl.gov/EisenSoftware.htm>) were used with the settings "complete linkage" and "uncentered correlation" as a similarity metric. No "self-organizing map" preclustering was performed. For the hierarchic cluster analysis, chromosomal gains were coded as 1 and chromosomal losses were coded as -1. To avoid overinterpretation of the data, only first- and second-generation clusters (i.e., the first- and second-level branches of the tree) were used for classification purposes.

Results

Comparative Genomic Hybridization results in relation to adenoma-carcinoma progression

Nineteen nonprogressed adenomas did not show any chromosomal abnormality. The remaining 47 nonprogressed adenoma cases showed few chromosomal aberrations (on average, 4.6). These aberrations showed a rather random distribution over the chromosomes, although there were

more losses than gains (ratio of loss to gain, 2:7). The average number of chromosomal aberrations in the 46 progressed adenomas was 10.5, significantly higher than in the nonprogressed adenomas ($P=0.000$) and similar to the value found in the carcinomas. Here losses and gains were more equally distributed (ratio of loss to gain, 1:3). Six chromosomal abnormalities occurred in more than 35% of cases: loss at 8p21-pter, 15q11-q21, 17p12-13, and 18q12-21 and gain at 8q23-qter and 13q14-31 (Table 2).

In all but 1 of 46 carcinoma parts of malignant polyps, and in all simple carcinomas, many chromosomal abnormalities were observed (average, 11.4 and 10.5, respectively). Seven chromosome changes occurred in more than 40% of cases. These were losses at 8p21-pter, 15q11-q21, 17p12-13, and 18q12-21 and gains at 8q23-qter, 13q14-31, and 20q13 (see Table 2). Because these events were associated with both the carcinomas and the adenomas that had already progressed to adenomas, they are further referred to as cancer-associated events. The distribution of these cancer-associated events over the different categories of lesions is depicted in Figure 1. The mean number of cancer-associated events correlated with adenoma-carcinoma progression, showing a significant difference in incidence between nonprogressed and progressed adenomas: 0.74 vs. 2.85 ($P=0.001$). The differences in mean number of cancer-associated events between progressed adenomas and their carcinoma counterparts (2.85 vs. 3.24) and between carcinoma parts of malignant polyps and simple carcinomas (3.24 vs. 3.36) were not statistically significant.

APC and KRAS in relation to adenoma-carcinoma progression

No significant differences in *APC* mutations were found between the groups; frequencies ranged from 54% in the simple adenomas to 71% in the carcinomas (Table 2). All mutations led to stop-codons, and thus to a truncated and inactive protein. *KRAS* mutations occurred more frequently (48%) in nonprogressed adenomas than in the other groups (28%–33%), with mostly G-T transition (8 of 12) in the nonprogressed adenomas as opposed to mainly G-A transition (10 of 15) in the progressed adenomas and the carcinomas.

Genetic data in relation to histopathologic characteristics

In the 112 adenomas, the number of CGH abnormalities was significantly correlated with grade of dysplasia ($P=0.002$), but not with histologic type or size (Table 2). The same was found for the number of cancer-associated events ($P=0.000$). Nevertheless, after stratification for grade of dysplasia, a significant difference in number of cancer-associated events between progressed and nonprogressed adenomas persisted (Figure 2). In addition, 2 specific chromosome changes – loss of 18q and gain of 20q – correlated with grade of dysplasia ($P=0.000$ for both).

Table 2. Results of genetic analyses of all tumors
A. Results of genetic analyses according to category and histologic grade of colorectal adenomas and carcinomas.

Category	Degree of dysplasia	CGH total	CGH loss /gain	ratio	CA events	Chromosomal aberrations (%)						K-ras mutation	APC mutation
						8p-	8q+	13q+	15q+	17p-	18q-		
Nonprogressed adenomas	All (n=66)	4.6	2.7	0.74	12	6	17	12	29	9	8	48% (12/25)	54% (22/41)
	Mild (n=20)	3.9	1.0	0.75	10	15	5	30	10	10	10	25% (3/12)	70% (7/10)
	Moderate (n=35)	4.3	5.6	0.66	14	0	11	14	26	9	6	82% (9/11)	54% (13/24)
	Severe (n=11)	6.7	2.7	1.00	9	9	36	18	36	9	9	0% (0/2)	29% (2/7)
Progressed adenomas	All (n=46)	10.5	1.3	2.85	39	35	50	35	57	50	30	28% (7/25)	60% (24/40)
	Mild (n=0)	-	-	-	-	-	-	-	-	-	-	-	-
	Moderate (n=26)	9.2	1.4	2.31	35	42	50	23	54	31	7	42% (5/12)	67% (14/21)
	Severe (n=20)	12.1	1.3	3.55	45	25	50	50	60	75	60	15% (2/13)	53% (10/19)
Differentiation grade		CGH total	CGH loss /gain	ratio	CA events	Chromosomal aberrations (%)						K-ras mutation	APC mutation
Carcinomas (malignant polyp)	All (n=46)	11.4	1.6	3.24	41	39	57	41	65	54	30	28% (7/25)	60% (24/40)
	Well (n=13)	10.5	1.5	3.00	39	23	62	54	54	39	39	22% (2/9)	50% (6/12)
	Moderate (n=31)	12.0	1.7	3.26	39	45	55	35	68	61	26	33% (5/15)	62% (16/26)
	Poor (n=2)	8.5	2.4	4.50	100	50	50	50	100	50	50	0% (0/1)	100% (2/2)
Carcinoma (simple)	All (n=36)	10.5	1.5	3.36	44	53	56	31	56	58	39	33% (1/3)	71% (10/14)
	Well (n=2)	14.5	1.1	4.00	50	50	50	50	100	50	50	-	-
	Moderate (n=31)	10.0	1.6	3.26	45	48	55	29	48	61	39	33% (1/3)	75% (9/12)
	Poor (n=3)	12.7	1.2	4.00	33	100	67	33	100	33	33	-	50% (1/2)

B. Results of genetic analyses in relation to category, size and histologic type of colorectal adenomas.

Category	Size	CGH total	CGH ratio	CA events	Chromosomal aberrations (%)							K-Ras mutation	APC mutation
					loss / gain	8p-	8q+	13q+	15q-	17p-	18q-		
Nonprogressed adenomas	<1 cm (n=31)	3.8	2.0	0.61	6	6	16	13	29	3	3	46% (6/13)	53% (10/19)
	1-2 cm (n=21)	5.3	3.3	0.86	19	9	14	10	24	19	14	50% (4/8)	62% (8/13)
	>2 cm (n=14)	5.2	3.3	0.86	14	0	21	14	36	7	7	50% (2/4)	44% (4/9)
Progressed adenomas	<1 cm (n=7)	6.0	0.9	2.14	14	57	43	29	43	14	0	0% (0/3)	71% (5/7)
	1-2 cm (n=6)	11.8	2.2	3.83	83	33	33	50	67	67	50	0% (0/4)	50% (3/6)
	>2 cm (n=33)	11.2	1.3	2.82	36	30	55	33	58	55	33	39% (7/18)	59% (16/27)
Histologic type		CGH total	CGH ratio	CA events	Chromosomal aberrations (%)							K-Ras mutation	APC mutation
Nonprogressed adenomas	Tubular (n=36)	3.7	2.3	0.58	8	8	14	8	25	8	6	25% (4/16)	65% (15/23)
	Tubulovillous (n=22)	5.2	3.1	0.95	9	0	27	18	36	9	9	83% (5/6)	43% (6/14)
	Villous (n=7)	7.3	2.9	1.00	43	14	0	14	29	14	14	100% (3/3)	33% (1/3)
Progressed adenomas	Tubular (n=15)	11.6	1.0	3.47	53	53	60	33	53	53	33	0% (0/7)	53% (8/15)
	Tubulovillous (n=28)	9.8	1.7	2.61	32	21	43	39	43	61	32	38% (6/16)	67% (16/24)
	Villous (n=2)	9.5	0.5	1.00	0	50	50	0	0	50	0	50% (1/2)	100% (1/1)

C. Results of genetic analyses in relation to Astler-Coller stage of colorectal carcinomas.

Category	Astler-Coller Stage	CGH total	CGH ratio	CA events	Chromosomal aberrations (%)							K-ras mutation	APC mutation
					loss / gain	8p-	8q+	13q+	15q-	17p-	18q-		
Carcinoma (malignant polyp)	Stage A (n=25)	10.8	2.1	3.56	40	44	60	56	72	60	40	20% (3/15)	73% (16/22)
	Stage B (n=11)	12.4	1.2	2.45	27	27	55	18	55	55	18	33% (2/6)	56% (5/9)
	Stage C,D (n=6)	10.2	2.2	2.83	50	17	50	33	67	50	17	50% (2/4)	20% (1/5)
Carcinoma (simple)	Stage A (n=1)	3.0	3.0	2.00	0	0	0	0	100	100	0	-	-
	Stage B (n=22)	10.5	1.8	2.95	46	36	59	27	50	50	27	50% (1/2)	73% (8/11)
	Stage C,D (n=11)	10.0	1.1	4.00	46	91	55	36	55	64	55	0% (0/1)	67% (2/3)

NOTE. Listed are the total number of chromosomal abnormalities (CGH total), the ratio between the number of losses and gains (ratio loss/gain), the number of cancer-associated events, the respective frequencies of these seven 'cancer-associated events': losses at 8p21-pter, 15q11-q21, 17p12-13, 18q12-21 and gains at 8q23-qter, 13q14-31 and 20q13; and the frequencies of K-ras and APC mutation. (The latter 2 are followed by the number of cases in which this was analyzed; CGH has been done on all cases). The data are listed according to category and grade of dysplasia and grade of differentiation (A); to category, size and histologic type of adenomas (B); and to category and Astler-Coller stage of carcinomas (C).

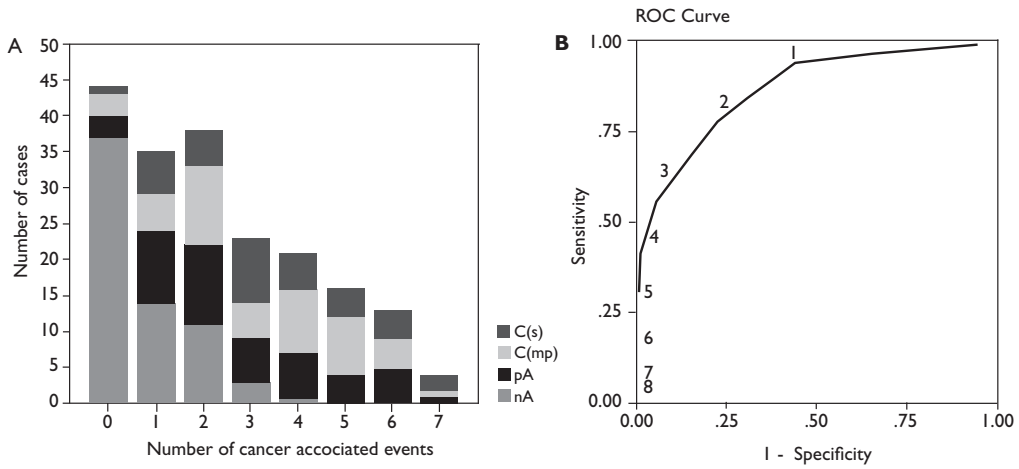


Figure 1. (A) Distribution of the number of cancer-associated events (losses at 8p21-pter, 15q11-q21, 17p12-13 and 18q12-21 and gains at 8q23-qter, 13q14-31, and 20q13) detected by CGH in 194 colorectal tumor samples. nA, nonprogressed adenoma; pA, progressed adenoma (i.e., adenoma part of a malignant polyp); C(mp), carcinoma part of a malignant polyp; C(s), simple carcinoma. **(B)** ROC curve showing the potential of the number of cancer-associated events (losses at 8p21-pter, 15q11-q21, 17p12-13, and 18q12-21 and gains at 8q23-qter, 13q14-31, and 20q13) detected by CGH in 194 colorectal tumor samples, to distinguish between progressed and nonprogressed lesions. The area under the curve is 0.87. Using a threshold of 2 cancer-associated events, the curve predicts 78% sensitivity with 78% specificity

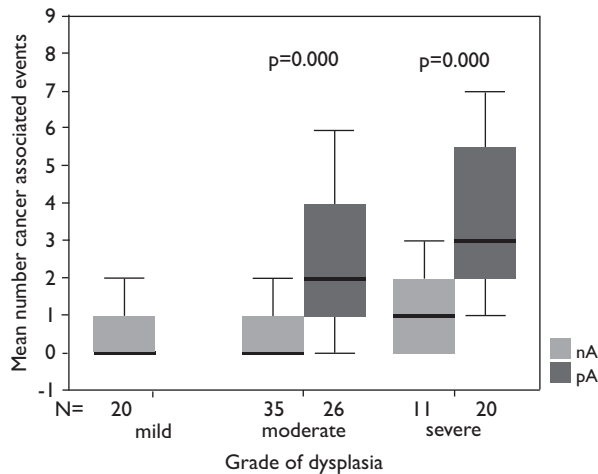


Figure 2. The number of cancer-associated events (losses at 8p21-pter, 15q11-q21, 17p12-13, and 18q12-21 and gains at 8q23-qter, 13q14-31, and 20q13) detected by CGH in 66 simple colorectal adenomas and 46 adenoma parts of malignant polyps. After stratifying for grade of dysplasia, adenoma parts of malignant polyps have significantly more cancer-associated events than simple adenomas.

Mutations in the *APC* gene did not correlate with grade of dysplasia, size, or histologic type of adenoma (Table 2), or to any CGH abnormality. *KRAS* mutations instead were associated with moderate dysplasia ($P=0.002$) and villous architecture ($P=0.008$), but not with adenoma size or the number of chromosomal abnormalities. An inverse correlation was found between *KRAS* mutation and 8q gain ($P=0.008$). In the 82 carcinomas (i.e., both carcinoma parts of malignant polyps as well as simple carcinomas), neither the CGH results nor *APC* or *KRAS* mutations correlated with degree of differentiation, size, or Astler-Coller stage (Table 2).

Comparative Genomic Hybridization results: hierarchic cluster analysis

Hierarchic cluster analysis of CGH results was performed in the 112 adenomas (i.e., 66 nonprogressed adenomas and 46 adenoma parts of malignant polyps), for ordering correlated groups of both tumors and chromosomal aberrations. This resulted in 4 clusters of colorectal adenomas (based on the first- and second-level branches of the tree), with distinctive patterns of chromosome changes (Figure 3A; Table 3). Adenomas in cluster 1 (AC1) showed no abnormalities. Adenomas in cluster 2 (AC2) had few abnormalities, consisting predominantly of losses, with loss of 17p and 20q as well as *KRAS* mutation (>40% for all 3) as main characteristics. In contrast, AC3 and AC4 showed a high number of both losses and gains. AC3 contained more than 40% gains of 2q, 5q, 6q, 7p+q, 8q, and 13q and loss of 17p, whereas AC4 showed more than 40% losses of 5q and 18q and gains of 12q and 20p+q. Certain associations between histologic type and the clustering results were observed (Table 3), whereas other parameters, including *APC* mutation and grade of dysplasia, did not differ between the clusters.

Hierarchic cluster analysis of the 82 carcinomas (i.e., both carcinoma parts of 46 malignant polyps as well as 36 simple carcinomas) yielded 2 first- and second-generation carcinoma clusters for further evaluation. (One first-generation cluster, containing only a single carcinoma without any chromosomal abnormalities, was excluded from further analysis.) These 2 clusters (CC1 and CC2) showed a similar distribution of carcinoma parts of malignant polyps and simple carcinomas, and no differences in degree of differentiation or Astler-Coller stage (Figure 3B and Table 3). CC1 had >40% losses of 11q, 12q, 17p, 17q, 18q, and 21q, whereas CC2 had >40% gains of 7p+q and 20q and loss of 18q. Gains of 8q and 13q occurred in more than 40% of both clusters. The chromosome abnormalities found in AC2 and AC4 matched with those found in CC1 and CC2, respectively, whereas AC3 appeared to be an intermediate group. This similarity was confirmed in an independent hierarchic cluster analysis run in which all 194 adenoma and carcinoma cases were entered together (Table 4).

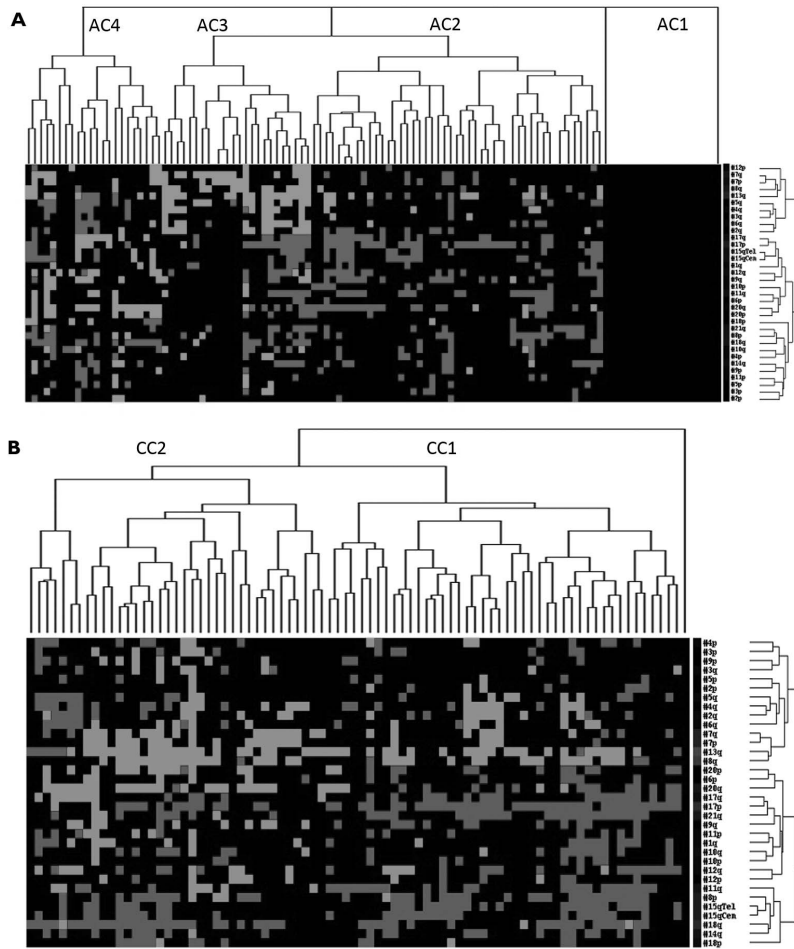


Figure 3. Heat map representations of the results of 2 separate hierarchic cluster analyses of (A) 112 colorectal adenomas and (B) 82 colorectal carcinomas, based on chromosomal gains and losses detected by CGH. Each row represents a specific chromosome arm, and every column represents a separate tumor. Green cells represent gains, red cells represent losses, and black cells indicate no abnormality. Rows are ordered according to correlations between chromosomal changes, following the tree displayed on the right side. Tumors are grouped on the basis of similarities in chromosomal aberrations following the tree displayed on top. Some chromosomal abnormalities are closely correlated, such as 8q and 13q gains, both in the (A) adenomas and in the (B) carcinomas. Only first- and second-generation tumor clusters (the first- and second-level branches of the tree) were used for classification purposes. In the adenoma tree, 4 clusters emerged, whereas in the carcinoma tree, 2 major clusters appeared (next to 1 carcinoma that did not show any chromosomal abnormality).

Table 3. Results of genetic analyses and histopathologic characteristics of all adenoma and carcinoma clusters.

	ac1 (n=19)	ac2 (n=47)	ac3 (n=24)	ac4 (n=22)	cc1 (n=44)	cc2 (n=37)
APC	45% (5/11)	53% (19/36)	65% (13/20)	64% (9/14)	66% (21/32)	59% (13/22)
K-ras	33% (3/9)	50% (12/24)	18% (2/11)	33% (2/6)	22% (5/23)	50% (2/4)
No CGH aberrations	0	7.0	9.8	10.1	11.0	11.3
% CGH gains	0	10%	57%	53%	25%	55%
% CGH loss	0	90%	43%	47%	75%	45%
Ratio loss/gain	-	9.0	0.8	0.9	3.0	0.8
% 11q-	0	40	29	9	52	8
% 12q-	0	38	29	9	43	14
% 17p- *	0	64	50	14	84	35
% 17q-	0	32	33	5	43	11
% 20q-	0	45	25	0	30	3
% 21q-	0	26	25	14	55	19
% 2q+	0	2	50	5	23	16
% 5q+	0	4	58	0	14	8
% 6q+	0	2	46	5	18	14
% 7p+	0	0	42	27	9	49
% 7q+	0	0	63	27	16	49
% 8q+ *	0	6	58	14	41	51
% 13q+ *	0	23	67	32	52	62
% 5q-	0	15	8	41	9	27
% 12q+	0	4	13	41	21	22
% 18q- *	0	17	29	64	43	73
% 20p+	0	2	0	41	5	35
% 20q+ *	0	6	4	68	11	62
nA	19	30	9	8		
pA	0	17	15	14		
C(mp)					27	18
C(s)					17	19
Size (mm)	13.4	20.7	20.1	28.8	33.8	37.7
Mild dysplasia	4	9	5	2		
Moderate dysplasia	11	27	13	10		
Severe dysplasia	4	11	6	10		
Tubular	13	19	13	6		
Tubulovillous	5	20	10	15		
Villous	1	6	1	1		
Well differentiated					11	4
Moderately differenti- ated					30	31
Poorly differentiated					3	2
Stage A					19	7
Stage B1,2					16	16
Stage C,D					8	9

NOTE. The adenomas and carcinomas have been clustered separately. Chromosome regions involved >40% in at least 1 of the clusters are presented. "Cancer-associated events" (see text) are marked with an asterisk (*).

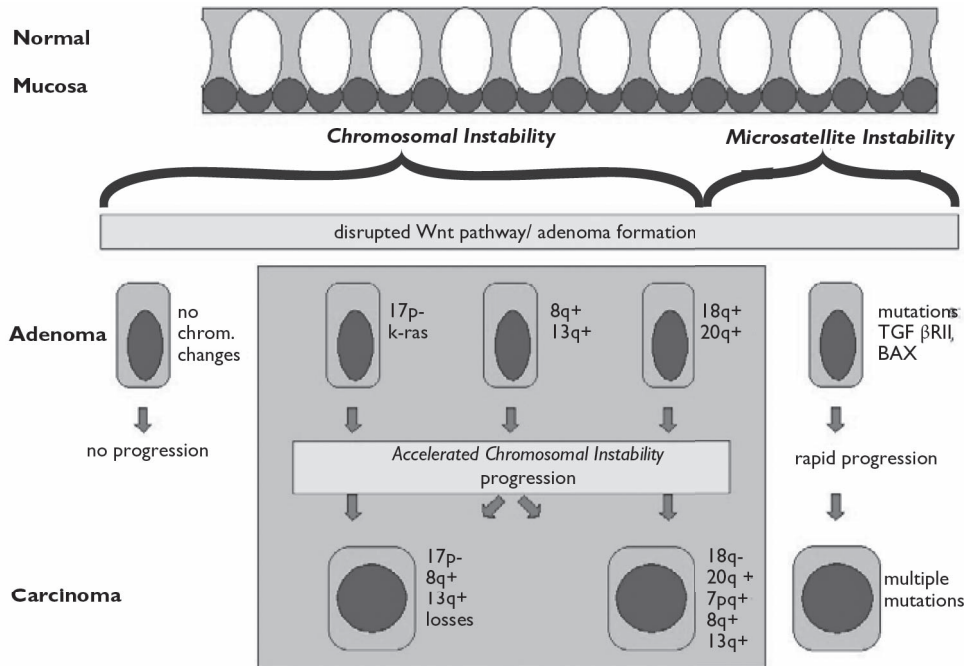


Figure 4. Chromosomal pathways in colorectal carcinogenesis. After the initiation of genomic instability, either as chromosomal instability or as microsatellite instability, disruption of the Wnt-signaling pathway causes adenoma formation. Progression from adenoma toward carcinoma in the chromosomal-liable group (which represents approximately 85% of all colorectal cancers) occurs in only about 5% of the adenomas and is associated with the acquisition of specific combinations of chromosomal changes.

Table 4. Cross table with the results of hierarchical cluster analysis of all 194 colorectal tumor samples together

	All tumors cluster 1	All tumors cluster 2	All tumors cluster 3
Adenoma cluster 1	19		
Adenoma cluster 2		47	
Carcinoma cluster 1		44	
Adenoma cluster 3		18	7
Adenoma cluster 4		5	17
Carcinoma cluster 2		7	30

NOTE. Vertical axis: clustering of 112 adenomas and of 82 carcinomas separately versus horizontal axis: clustering of all 194 tumors together. The separate clustering yielded 4 adenoma clusters and 2 carcinoma clusters; the latter resulted in three clusters; the clustering of all tumor cases together resulted in 3 all tumor clusters. The all tumors cluster 1 cases showed no chromosomal aberrations and completely matched with adenoma cluster 1. All cases in adenoma cluster 2 and carcinoma cluster 1 completely were assigned to all tumors cluster 2, whereas 80% (47 out of 59) of adenoma cluster 4 and carcinoma cluster 2 cosegregated with all tumors cluster 3. Cases from adenoma cluster 3 distributed all over cluster 2 (72%) and all tumors cluster 3 (28%).

Discussion

Current genetic models of colorectal adenomacarcinoma progression do not reflect the complexity of the disease. We compared genomewide chromosomal aberrations in nonprogressed adenomas to adenomas proven capable of progression toward malignancy. The present data, obtained from a cross-sectional study of a total of 194 tumor samples, revealed that gains at 8q23-qter, 13q14-31, and 20q13 and losses at 8p21-pter, 15q11-q21, 17p12-13, and 18q12-21 were strongly associated with advanced lesions, including both adenomas that contained foci of carcinoma and simple carcinomas. These former lesions implicitly demonstrated their capability of progression. The presence of these “cancer-associated events” in colorectal adenoma thus could be an indicator of high risk of progression. In addition, the present study yielded evidence for the association of multiple independent patterns of chromosomal aberrations associated with progressed adenomas and colorectal carcinomas, suggesting the presence of multiple chromosomal instability pathways toward colorectal cancer.

The accumulation of cancer-associated events correlated with progression from simple adenoma to adenomas with foci of cancer (malignant polyps). Surprisingly, after stratifying for dysplasia, progressed adenomas (i.e., adenomas containing a focus of carcinoma) had a significantly higher number of cancer-associated events than did nonprogressed adenomas with the same grade of dysplasia (Figure 2), indicating that in this respect genetic characteristics are more informative than morphologic characteristics.

The presence of cancer-associated events turned out to be a relatively strong indicator of progression toward malignancy in colorectal adenomas. Because screening strategies should detect adenomas that are actually likely to progress rather than the highly prevalent, indolent type of adenomas that do not progress, the reported cancer-associated events could become clinically important. The mean number of cancer-associated events appears to be a good discriminator between progressed and nonprogressed lesions. Putting the data in a receiver operator characteristic (ROC) curve, an area under the curve of 87% emerged, and when using the presence of 2 cancer-associated events as cutoff, a 78% sensitivity with a 78% specificity was achieved (Figure 1B). This is comparable to, or even better than, for example, the performance of fecal occult blood testing [17]. Figure 1 shows that indeed the vast majority of lesions with 2 or more cancer-associated events were progressed lesions. However, only a minority of progressed lesions had 4 or more cancer-associated events, which may indicate that it is not the mere accumulation that is important in carcinogenesis, but rather the acquisition of some specific combinations of events.

Indeed, hierarchic cluster analysis revealed the presence of specific combinations of alterations. Of the 7 cancer-associated events, 5 segregated over 3 distinct adenoma clusters (Table 3). The

cancer-associated events showed positive correlations within each cluster (i.e., 8q gain with 13q gain in AC3 and 18q loss with 20q gain in AC4), but inverse correlations between the clusters (i.e., 8q/13q gain with 18q loss/20q gain and 17p loss with 18q loss/20q gain). In addition, *KRAS* mutation, common in AC2 together with 17p loss, showed inverse correlation with the abnormalities of AC3 and AC4, that is, to 8q/13q gain and 18q loss/20q gain, respectively. The persistence of such combinations of chromosomal aberrations in separate clusters of adenomas and carcinomas, and their mutually exclusive nature, suggest that specific combinations, rather than a mere accumulation of genetic abnormalities, are essential for progression [18]. Interestingly, a separate clustering of the 82 carcinomas resulted in 2 clusters that showed great similarity with 2 of the adenoma clusters. This was confirmed when all adenomas and carcinomas were clustered together (Table 4). These data are compatible with the existence of multiple routes of progression through chromosomal instability (Figure 4).

These data show that chromosomal instability does not cause merely random genetic noise, but instead distinct, statistically highly significant patterns of associated chromosomal changes. In the end, the functional consequences of these chromosomal aberrations on gene expression level are what cause tumor progression. As a consequence, the respective chromosomal regions are likely to harbor gatekeeper genes involved in different cell biologic processes, such as proliferation, apoptosis, invasion, remodeling of extracellular matrix, stroma induction, and escape from the immune system [19]. Comparison of microsatellite-unstable and chromosomal-unstable colorectal cancers supports the concept of specific molecular pathways being disrupted in different ways. Although in microsatellite-unstable colorectal cancers the balance of proliferation and differentiation is altered by *TGFβRII* mutation [20] and apoptosis is altered by *BAX* mutation [21], in chromosomal-unstable colorectal cancers, the same pathways can be impaired via 18q loss (*Smad4*) and 17p loss (*p53*) [20-21].

The present data indicate that even within the group of chromosomal-unstable colorectal tumors, different ways may exist to affect the same cellular functions. The most obvious candidate genes that are compatible with the chromosomal changes found are *p53* on 17p12 and *Smad4* on 18q21. For the other cancer-associated events, this remains to be determined. Further studies are needed to elucidate which critical molecular pathways are affected by these chromosomal alterations, and how these contribute to colorectal adenoma-carcinoma progression.

Our data are consistent with the recent finding that a certain degree of genomic instability is already present in very small (i.e., early) adenomas [22-24]. In small, nonprogressed adenomas, chromosomal aberrations were sometimes found, mostly losses. Disruption of the Wnt pathway, frequently by loss of APC function, is the earliest factor causing the development of colorectal adenomas [25], and in mouse models APC mutation has been found to be associated with

chromosomal instability [26,27]. However, because *APC* mutations occur in the vast majority of adenomas (including microsatellite-unstable adenomas that have normal karyotypes [28], and because only about 5% of these adenomas ever progress to cancer, it is unlikely that loss of *APC* function is the sole determining factor in chromosomal instability in colorectal adenomas. Indeed, in our study, the increase of chromosomal aberrations found to be associated with adenoma-carcinoma progression was not correlated with the presence of *APC* mutations. Therefore, other factors must be involved in accelerating genomic instability [29].

We conclude that the acquisition of 2 or more cancer-associated events is associated with progressed colorectal adenomas and carcinomas. In addition, this association involves specific combinations of a few chromosomal abnormalities, rather than the mere accumulation of events. Evidence is shown for the existence of multiple chromosomal instability pathways of colorectal cancer progression, each of which may possibly disrupt similar cell biologic functions, such as proliferation, differentiation, apoptosis, and invasion, in different manners.

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