Analysis of chromosomal instability in human colorectal adenomas with two mutational hits at *APC*

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***In vitro* data show that the adenomatous polyposis coli (APC) protein associates with the mitotic spindle and that mouse embryonic stem cells with biallelic *Apc* mutations are karyotypically unstable. These findings led to suggestions that APC acts in chromosomal segregation and that APC inactivation leads to chromosomal instability (CIN). An alternative hypothesis based on allelic loss studies in colorectal adenomas proposes that CIN precedes and contributes to genetic changes at *APC*. We determined whether colorectal adenomas with two mutations at *APC* show features consistent with these models by studying 55 lesions (average size 5 mm; range 1–13 mm) from patients with familial adenomatous polyposis. A variety of methods was used depending on available material, including flow cytometry, comparative genomic hybridization, and loss of heterozygosity (LOH) analysis. Selected adenomas were assessed for proliferative activity by Ki-67 immunocytochemistry. Seventeen of 20 (85%) tumors were diploid, two were near-diploid, and one was hypotetraploid. Just one (near-diploid) tumor showed increased proliferative activity. LOH was found occasionally on chromosome 15q (2 of 49 tumors), but not on chromosome 18q (0 of 48). In 20 adenomas, LOH at *APC* was associated with loss at 5q but not 5p markers, with the former encompassing a minimum of 20 Mb. However, three of these lesions analyzed by comparative genomic hybridization displayed normal profiles, suggesting, together with other data, that the mechanism of LOH at *APC* is probably somatic recombination. Our results therefore do not support the hypothesis that CIN precedes *APC* mutations in tumorigenesis. Regarding the model in which *APC* mutations lead directly to CIN, if *APC* mutations do have this effect *in vivo*, it must be subtle. Alternatively, CIN associated with *APC* mutations might be essentially an *in vitro* phenomenon.**

Most colorectal cancers, many sporadic adenomas, and all polyps in familial adenomatous polyposis (FAP) carry mutations in the *adenomatous polyposis coli* (*APC*) gene. *APC* encodes a 2,843-aa protein that is involved in several cellular processes, including the regulation of β-catenin, and that includes domains for binding to microtubules (reviewed in refs. 1 and 2). Disease-associated *APC* mutations fully or partially inactivate APC function, almost always by producing a truncated or absent protein (3–5). Truncated proteins are usually disrupted within their β-catenin binding/degradation domains and almost invariably lack the microtubule-binding sites, which are located at the C-terminal region of the molecule (6–10).

It is generally believed that *APC* mutations are selected, at least in part, for their effects on β-catenin levels (11–15): mutant APC cannot degrade β-catenin, leading to constitutive activation of the Wnt signaling pathway and to direct expansion of the mutant clone. The evidence that this effect of APC is critical has been derived from several sources, one being that colorectal tumors without *APC* mutations sometimes harbor mutations of β-catenin that prevent protein destruction and thus have effects similar to mutation of *APC* (16–18). It is unlikely, however, that inactivating *APC* mutations and activating β-catenin mutations are functionally identical, given, for example, that the former seem to be associated with a higher probability of progression from colorectal adenoma to carcinoma (19). Several groups have suggested, therefore, that loss of C-terminal APC functions provides a selective advantage additional to that which arises from constitutive Wnt signaling (20–24).

Fodde *et al.* (23) and Kaplan *et al.* (24) independently reported that APC may have a role in chromosomal segregation. Both groups studied mouse embryonic stem (ES) cells homozygous for a truncating *Apc* mutation (*Min* ES cells) and detected a marked increase in numerical and structural chromosome aberrations, as well as disorganized spindle microtubules. During mitosis, wild-type APC was found to be localized along kinetochore microtubules and at their ends adjacent to kinetochores. Together, these observations led the authors to suggest a new role for APC in kinetochore microtubule-chromosome attachment and therefore chromosome segregation, with mutations in *Apc* disrupting this function and resulting in chromosomal (or karyotypic) instability (CIN). In this way, *APC* mutations may be selected not only directly—through their effects on the Wnt pathway—but also indirectly, through the hypermutation which they engender in the form of CIN.

Shih *et al.* (25) analyzed 32 sporadic colorectal adenomas for loss of heterozygosity (LOH) by using digital single nucleotide polymorphism PCR and found relatively high frequencies of LOH on chromosomes 5q (55%), 1p (10%), 8p (19%), 15q (28%), and 18q (28%). Although digital SNP-PCR may provide some increase in sensitivity over microsatellite-based methods, owing to its use of confidence interval thresholds for scoring LOH, in reality most tumors with LOH in Shih *et al.*'s study (25) showed one of the SNP alleles to be at a frequency of 66% or more. This finding is equivalent to an allelic ratio of 2:1, the usual threshold for scoring LOH by using microsatellites. Most cases of LOH detectable by using digital SNP-PCR should, therefore, also be detectable by using microsatellite-based LOH. Although several explanations for their findings were discussed, Shih *et al.*'s (25) preferred interpretation of their data was that karyotypic instability occurred early in colorectal tumorigenesis, preceding and leading to *APC* mutations.

Abbreviations: APC, adenomatous polyposis coli; CIN, chromosomal instability; FAP, familial adenomatous polyposis; FCM, flow cytometry; CGH, comparative genomic hybridization; LOH, loss of heterozygosity; PI, propidium iodide; DI, DNA index.

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Evidently, the findings of Fodde *et al.* (23) and Kaplan *et al.* (24) using *in vitro* methods may not apply *in vivo*, and Shih *et al.* (25) made no direct assessment of chromosomal-scale changes in their tumors. We have, therefore, studied a set of 55 colorectal adenomas (average size = 5 mm; range = 1–13 mm) from 18 FAP patients with a variety of germ-line mutations and characterized second hits at *APC*. Using a variety of analytical methods [flow cytometry (FCM), LOH analysis, and comparative genomic hybridization (CGH)], we searched for evidence of aneuploidy and polyploidy in these tumors. In addition, Ki-67 immunocytochemistry was used to assess the proliferative activity in a subset of these lesions. To elucidate the molecular mechanism underlying allelic loss at *APC*, the extent of LOH on chromosome 5 was determined in 20 adenomas.

Patients and Methods

Study Population. This study examined 55 colorectal adenomas and four normal biopsies from 18 patients diagnosed with FAP and with a known germ-line *APC* mutation. All tumors were tubular adenomas with mild dysplasia (average size = 5 mm; range = 1–13 mm) that had either been fresh-frozen at colectomy ($n = 47$) or fixed in formalin and embedded in paraffin ($n = 8$). A minimum of 60% neoplastic material was present in each biopsy as assessed by the analysis of hematoxylin and eosin-stained sections. The second hit at *APC* had been determined in all lesions by using standard mutation detection techniques [single-strand conformation polymorphism (SSCP) analysis, DNA sequencing] and LOH analysis at microsatellite markers close to the *APC* locus (D5S346, D5S656, and D5S421). Details of the colorectal adenomas are summarized in Table 1.

FCM and Ki-67 Immunocytochemistry. Multiparameter FCM was performed on 4 paraffin-embedded and 16 fresh-frozen tumors, as well as 2 respective normal biopsies. All fresh-frozen tissue was simultaneously assayed for expression of the Ki-67 antigen by using FITC-labeled monoclonal mouse antibody (Dako). The appropriate FITC-labeled mouse IgG1 antibody (Dako) was used as isotype control. In brief, a small piece (about 4 mm^3) of fresh-frozen tissue was disaggregated into a cellular suspension by using the Dako Medimachine System. Cells were fixed in 70% ethanol for 40 min at 4°C, washed twice in PBS, 0.5% Tween 20 (pH 7.2), and resuspended in 80 μl of PBS, 0.5% Tween 20, 0.5% BSA (pH 7.2). Incubation with 20 μl of antibody was performed for 30 min at 4°C. Cells were washed twice in PBS, 0.5% Tween 20, 0.5% BSA (pH 7.2), treated with 100 μg/ml RNase, and stained with 50 μg/ml propidium iodide (PI). The suspension was filtered through a 70-μm nylon filter and immediately analyzed on a FACSCalibur (Becton Dickinson). Cells were excited by the argon laser emitting at 488 nm. Fluorescence from FITC-labeled antibodies was detected by using a 530/30-nm band pass filter, and PI fluorescence was detected by using a 670-nm long pass filter. Forward and right angle light scatter were used to set a gate including all cells, but excluding debris. A second gate set on area and width of PI fluorescence was used to further define the single cell population. Both FITC and PI fluorescence were collected in linear mode, and acquisition was stopped after 8,000 gated events had been acquired. Data were analyzed for proliferative activity (percentage of Ki-67-positive cells normalized against the isotype control) and aneuploidy [DNA index (DI)] by using dedicated MODFIT software (Verity Software House, Topsham, ME). The DNA diploid peak was set by using the two normal samples. Paraffin-embedded tissue was prepared for analysis by cutting a 50-μm section from each block, placing it into a histopathology cassette between two sheets of Whatman 3MM filter paper, and dewaxing it in xylene overnight. The section was rehydrated in an ethanol series and rinsed twice in water. The tissue was digested in 0.4% pepsin for 30 min at 37°C, and the digestion was stopped in 0.2% glycine, 2× PBS (pH 7.2). Cells were washed twice in PBS (pH 7.2) before FCM analysis.

LOH (Allelic Loss) Analysis. LOH analysis was performed at microsatellite markers on chromosome 15q (D15S995 and D15S1007; close to the *CRAC1* locus) and chromosome 18q (D18S46 and D18S470; close to the *SMAD4/MADH4* locus). In 20 adenomas with LOH as the second hit at *APC*, the extent of allelic loss on chromosome 5 was determined by using six microsatellite markers (D5S2845, 5p14.3; D5S1470, 5p13.3; D5S82, 5q21.3; D5S489, 5q22.3; D5S2117, 5q31.1; and D5S1456, 5q35.1). Standard methods of fluorescence-based genotyping were used on the ABI377 (Applied Biosystems) semiautomated sequencer. Allelic loss was scored if the area under one allelic peak in the tumor was reduced by 50% or more relative to the other allele, after correcting for the relative peak areas by using normal DNA.

CGH. CGH was performed on five fresh-frozen tumors containing at least 60% neoplastic material, as described (26). Briefly, 50–100 ng of tumor and reference DNA were amplified by degenerate oligonucleotide-primed PCR (DOP-PCR) and fluorescently labeled by nick translation (27). Labeled tumor and normal DNA were precipitated in the presence of 50 μg Cot1 DNA (Life Technologies, Grand Island, NY) and dissolved in hybridization buffer (50% formamide/10% dextran sulfate/2× SSC). The mixture was denatured at 75°C for 5 min, left to preanneal for 30 min, and applied to denatured metaphase spreads (Vysis, Downers Grove, IL) prepared from normal male peripheral blood lymphocytes. The metaphase spreads were denatured in 70% formamide, 2× SSC at 73°C and dehydrated in an ethanol series. Slides were left to hybridize for 2–3 days at 37°C, then washed in 50% formamide, 2× SSC, followed by a wash in 2× SSC. After air drying, the slides were counterstained with 4,6-diamino-2-phenylindole (DAPI). Images were captured with a charge-coupled device (CCD) camera attached to a Zeiss axioskop microscope and analyzed by using QUIPS (Vysis, Downers Grove, IL) software. Between 5 to 10 metaphases were analyzed for each tumor. Negative control hybridizations were included in each batch of experiments. A chromosomal region was considered to be lost or gained if the mean hybridization ratio between tumor and normal DNA was $<0.85:1$ or $>1.15:1$, respectively (26).

Results

A total of 55 colorectal adenomas with two characterized mutational hits at *APC* was analyzed for CIN. A variety of techniques was used, depending on the amount and type of material available. Where possible, the proliferative activity of these lesions was also assessed.

Four paraffin-embedded and 16 fresh-frozen tumors were analyzed for aneuploidy/polyploidy by multiparameter FCM (Table 2). Three of 20 (15%) adenomas contained subpopulations of cells displaying changes in ploidy, with two being near-diploid (DI = 1.2 and DI = 0.8) and one being hypotetraploid (DI = 1.8) (Fig. 1). In addition, Ki-67 immunocytochemistry was performed on all fresh-frozen lesions, as well as two normal biopsies, to assess their proliferative activity (percentage of Ki-67-positive cells). No apparent difference in the proportion of Ki-67-expressing cells was observed between diploid tumors and normal colonic tissue. However, one of the two aneuploid polyps studied displayed an increase in the proportion of Ki-67-expressing cells (53.1%), as compared with the euploid biopsies (average = 16.8%; range = 5.0–35.7%).

Table 1. Patient ID, *APC* mutation status, and size of the colorectal adenomas analyzed in this study, as well as analytical methods applied

Patient ID	Adenoma ID	Germ line *APC* mutation (nucleotide; codon)	Somatic *APC* mutation (nucleotide; codon)	Adenoma size, mm	Analytical method
N-1144	249a	502 A>T; R168X	4192 del 2bp; 1398 FS	4	L15/18
	249b	502 A>T; R168X	4316 del 1bp; 1439 FS	3.5	L15/18
	315	502 A>T; R168X	4132 C>T; Q1371X	5.5	L15/18, FCM, Ki-67
N-1154	203	1495 C>T; R499X	4466 ins 2bp; 1489 FS	4.5	L15/18, FCM, Ki-67
	298	1495 C>T; R499X	4393 del 2bp; 1465 FS	8	L15/18, FCM, Ki-67
	300	1495 C>T; R499X	4012 C>T; Q1338X	6	L15/18
	301	1495 C>T; R499X	4317 del 1bp; 1439 FS	5	L15/18, FCM, Ki-67
	312	1495 C>T; R499X	3927 del 5bp; 1309 FS	7.5	L15/18, FCM, Ki-67, CGH
	340	1495 C>T; R499X	3916 G>T, E1306X	5	L15/18, FCM, Ki-67
	352	1495 C>T; R499X	4348 del 4bp; 1450 FS	5	L15/18, FCM, Ki-67, CGH
	240	1495 C>T; R499X	3927 del 5bp; 1309 FS	6.5	L15/18, FCM, Ki-67
	243	1495 C>T; R499X	3927 del 5bp; 1309 FS	5.5	L15/18
	259	1495 C>T; R499X	4216 C>T; Q1406X	6	L15/18, FCM, Ki-67
	264	1495 C>T; R499X	3927 del 5bp; 1309 FS	5	L15/18
	350	1495 C>T; R499X	4277 del 1bp; 1426 FS	6	L15/18, FCM, Ki-67
	374	1495 C>T; R499X	4466 del 1bp; 1489 FS	5	L15/18, FCM, Ki-67
	194b	1495 C>T; R499X	4316 del 1bp; 1439 FS	6	L15/18
N-117	155a	1842 ins 1 bp; 614 FS	4484 ins 1bp; 1495 FS	2.5	L15/18
	155b	1842 ins 1 bp; 614 FS	4306 del 13bp; 1436 FS	2.5	L15/18
	155c	1842 ins 1 bp; 614 FS	4446 del 10bp; 1482 FS	1	L15/18
N-1263	17	3183 del 5 bp; 1061 FS	4312 del 1bp; 1438 FS	2	L15/18
	135b	3183 del 5 bp; 1061 FS	3905 del 1bp; 1302 FS	1.5	L15/18
N-1016	308	3863 del 1bp; 1287 FS	LOH	5.5	FCM, Ki-67
N-609	1	3887 ins 13 bp; 1296 FS	LOH	3	L15/18
	2	3887 ins 13 bp; 1296 FS	LOH	3	L15/18
	5	3887 ins 13 bp; 1296 FS	LOH	3	L15/18
	6	3887 ins 13 bp; 1296 FS	LOH	3	L15/18
	8	3887 ins 13 bp; 1296 FS	LOH	3	L15/18
	10	3887 ins 13 bp; 1296 FS	LOH	3	L15/18
N-1026	258	3907 C>T; Q1303X	LOH	4.5	L15/18, FCM, Ki-67
	335	3907 C>T; Q1303X	LOH	6	L15/18, FCM, Ki-67
N-283	347	3927 del 5bp; 1309 FS	LOH	4.5	L15, FCM, Ki-67
N-1066	187	3927 del 5bp; 1309 FS	LOH	7	L15/18
N-1633	292	3927 del 5bp; 1309 FS	LOH	6	L15/18
N-220	399	3927 del 5bp; 1309 FS	LOH	7	FCM, Ki-67
N-127	128a	3927 del 5bp; 1309 FS	LOH	5	L15/18
	128b	3927 del 5bp; 1309 FS	LOH	5	L15/18
	128c	3927 del 5bp; 1309 FS	LOH	5	L15/18, CGH
	129	3927 del 5bp; 1309 FS	LOH	1	L15/18, CGH
	130	3927 del 5bp; 1309 FS	LOH	5	L15/18
	131b	3927 del 5bp; 1309 FS	LOH	3	L15/18
	131c	3927 del 5bp; 1309 FS	LOH	3	L15/18, CGH
	131d	3927 del 5bp; 1309 FS	LOH	3	L15/18
N-907	3	3927 del 5bp; 1309 FS	LOH	3	L15/18
	5	3927 del 5bp; 1309 FS	LOH	3	L15/18
	8	3927 del 5bp; 1309 FS	LOH	3	L15/18
N-205	206	3927 del 5bp; 1309 FS	LOH	1	L15/18
	207	3927 del 5bp; 1309 FS	LOH	1	L15/18
	208	3927 del 5bp; 1309 FS	LOH	1	L15/18
	209	3927 del 5bp; 1309 FS	LOH	1	L15/18
	1974/92/B	3927 del 5bp; 1309 FS	LOH	11	FCM
N-458	1	3927 del 5bp; 1309 FS	LOH	12	FCM
52701	2929-M	3927 del 5bp; 1309 FS	LOH	6	FCM
N-610	5	3927 del 5bp; 1309 FS	LOH	12.5	FCM
N-351	2	4392 del 2bp; 1464 FS	LOH	3	L15/18

L15/18, LOH analysis at chromosomes 15q/18q; FCM, flow cytometry; Ki-67, Ki-67 immunocytochemistry; CGH, comparative genomic hybridization. FS denotes frameshift mutations.

Results were of similar quality for paraffin-embedded and fresh-frozen material, with the mean coefficient of variation (CV) of the G_1/G_0 peak being 4.9 ± 1.2 and 4.1 ± 0.5, respectively.

LOH analysis was performed on DNA from 49 adenomas. Importantly, all of these lesions contained <40% contaminating normal tissue, and 27 had shown unequivocal LOH as the second hit at *APC*. We did detect LOH on other chromosomes in these

Table 2. Results of flow cytometry on colorectal adenomas with two mutational hits at *APC* and two normal controls

Adenoma ID	DI	CV	Percentage of Ki-67 positive cells
Normal (N-283)	1.0	3.7	21.3
Normal (N-220)	1.0	4.39	11.6
315	1.0	4.07	15.2
203	1.0	3.81	21.6
298	0.8	3.54	29.2
301	1.0	5.32	26.1
312	1.0	3.68	20.8
340	1.0	4.26	11.0
352	1.0	4.29	14.5
240	1.0	3.8	7.0
259	1.0	4.14	14.2
350	1.0	3.45	5.0
374	1.0	4.56	6.3
308	1.0	4.29	17.2
258	1.0	4.25	18.3
335	1.2	3.19	53.1
347	1.0	3.57	35.7
399	1.0	4.42	22.2
1974/92/B	1.0	4.9	—
1	1.0	5.35	—
2929-M	1.8	2.96	—
5	1.0	6.52	—

DI, DNA index; CV, coefficient of variation of the G_1/G_0 peak; —, no data.

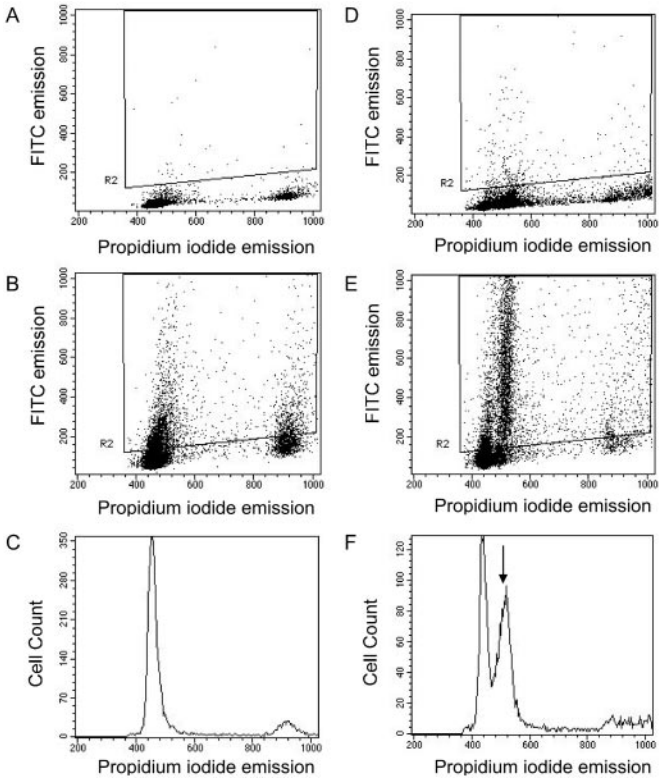

Fig. 1. Representative results of flow cytometry on one normal biopsy (N-283; *A–C*) and one near-diploid colorectal adenoma with two mutational hits at *APC* (335; *D–F*). (*A* and *D*) PI fluorescence against Ki-67-FITC fluorescence of the isotype control sample. The box represents Ki-67-positive events. (*B* and *E*) PI fluorescence against Ki-67-FITC fluorescence for the monoclonal antibody-stained samples. (*C* and *F*) The PI histograms. The near-diploid subpopulation is indicated by the arrow.

adenomas, but at a low frequency: although 2 of 49 (4%) informative tumors showed LOH at markers on chromosome 15q, none of 48 (0%) showed LOH at markers on chromosome 18q. Our previously published data had shown evidence of LOH on chromosome 1p in 1 of 21 (5%) of these polyps (28). No evidence of microsatellite instability (MSI+) was found at any marker in any polyp.

The extent of allelic loss on chromosome 5 was determined in 20 adenomas with LOH as the second hit at *APC* (Table 3). Nineteen (95%) tumors showed LOH at all informative markers spanning chromosome 5q, with the minimal detectable region of allelic loss encompassing ≈20 Mb. The remaining tumor displayed a normal dosage at the telomeric marker D5S2117. In contrast, none of the 20 adenomas showed allelic loss at markers on chromosome 5p.

CGH analysis on five fresh-frozen tumors, two of which had been shown to be euploid by FCM, revealed normal CGH profiles (Fig. 2). Interestingly, three of the polyps had shown LOH at *APC*, with allelic loss involving at least 20 Mb of chromosome 5q (Table 3). One of these polyps had also shown LOH on chromosome 15q. Although the region of loss at 15q may have been below the resolution of CGH, the failure to detect a deletion of chromosome 5q is in accordance with our real-time quantitative multiplex PCR (RQM-PCR) results, showing that LOH at *APC* does not result from physical loss of material (unpublished data).

Discussion

Our results using a variety of experimental methods show that the majority of adenomas with two mutational hits at *APC* are diploid or near-diploid. Just 3 of 20 (15%) tumors displayed evidence of chromosomal (or karyotypic) instability as determined by FCM and CGH analysis, two being near-diploid and one being hypotetraploid. Three further samples showed normal DNA profiles by CGH analysis, indicating an absence of gross unbalanced karyotypic rearrangements. In our samples, CIN was not associated with the type of second hit (truncating mutation or LOH) at *APC*. One of the two near-diploid lesions showed an increase in the proportion of Ki-67-expressing cells, as compared with all euploid biopsies. Given that models of colorectal tumorigenesis predict increasingly aggressive features as tumors progress, it is likely that this tumor had become aneuploid as part of its progression rather than as a direct result of inactivation of APC.

Complementing these findings, we have previously identified two mutational hits at *APC* in near-diploid, microsatellite unstable (MSI+) colorectal cancer cell lines with only a few (<5) chromosomal rearrangements (2, 7, 29). Two of these cell lines (LoVo and VACO5) harbored biallelic truncating *APC* mutations, and two showed a truncating *APC* mutation and LOH (DLD1 and GP2d/GP5d).

Apart from at the *APC* locus, LOH was uncommon in our polyps, being found at a frequency of ≈5% at chromosomes 1p (28) and 15q, but being absent at chromosome 18q. In 20 analyzed adenomas, allelic loss at *APC* was associated with loss at markers on chromosome 5q but not 5p, with the minimal region of allelic loss encompassing ≈20 Mb. CGH analysis of three of these polyps, however, revealed no unbalanced chromosomal rearrangements. Together with our real-time quantitative multiplex (RQM)-PCR findings on colorectal adenomas showing that LOH at *APC* does not result from deletion of material (unpublished data), these data suggest that the molecular mechanism of allelic loss at *APC* is nearly always somatic recombination.

Our data are in agreement with a recent report by Haigis *et al.* (30), who analyzed 18 colorectal polyps from Apc^{Min} mice

GENETICS

Table 3. Results of LOH analysis on colorectal adenomas with allelic loss as the second mutational hit at *APC*, using microsatellite markers spanning chromosome 5

Patient ID	Adenoma ID	D5S2845 5p14.3	D5S1470 5p13.3	D5S82 5q21.3	*APC* 5q22.2 D5S346	*APC* 5q22.2 D5S656	*APC* 5q22.2 D5S421	D5S489 5q22.3	D5S2117 5q31.1	D5S1456 5q35.1
N-127	128a	NL	NL	NI	NI	LOH	LOH	LOH	LOH	NI
	128b	NL	NL	NI	NI	LOH	LOH	LOH	LOH	NI
	128c	NL	NL	NI	NI	LOH	LOH	LOH	LOH	NI
	129	NL	NL	NI	NI	LOH	LOH	LOH	LOH	NI
	130	NL	NL	NI	NI	LOH	LOH	LOH	NL	NI
	131b	NL	NL	NI	NI	LOH	LOH	LOH	LOH	NI
	131c	NL	NL	NI	NI	LOH	LOH	LOH	LOH	NI
	131d	NL	NL	NI	NI	LOH	LOH	LOH	LOH	NI
N-907	3	NL	NL	LOH	LOH	LOH	LOH	LOH	LOH	LOH
	5	NL	NL	LOH	LOH	LOH	LOH	LOH	LOH	LOH
	8	NL	NL	LOH	LOH	LOH	LOH	LOH	LOH	LOH
N-609	1	NL	—	LOH	LOH	LOH	LOH	LOH	LOH	LOH
	2	NL	NL	LOH	LOH	LOH	LOH	LOH	LOH	LOH
	5	NL	NL	—	LOH	LOH	LOH	LOH	LOH	LOH
	6	NL	NL	LOH	—	LOH	LOH	LOH	LOH	LOH
	8	NL	NL	LOH	LOH	LOH	LOH	LOH	LOH	LOH
	10	NL	NL	LOH	LOH	LOH	LOH	LOH	LOH	LOH
N-205	207	NL	NL	LOH	LOH	LOH	LOH	LOH	LOH	LOH
	208	NL	NL	—	LOH	LOH	LOH	LOH	LOH	LOH
	209	NL	NL	—	LOH	LOH	LOH	LOH	LOH	LOH

NI, noninformative; NL, no loss; —, no data. LOH at *APC* has been scored based on three markers in close proximity to the locus, D5S346, D5S656, and D5S421.

and six human adenomas from patients without FAP that had uncertain *APC* mutation status. Haigis *et al.* (30) used interphase fluorescence *in situ* hybridization (FISH) analysis on selected mouse/human chromosomes and found no evidence of chromosomal gains or losses. In tumors from Apc^{Min} mice, this result was confirmed by karyotypic analysis. Furthermore, allelic loss at *Apc* was shown to commonly occur by somatic recombination in *Min* adenomas. Other studies have found early colorectal adenomas to be near-diploid in most cases, although larger and/or more dysplastic lesions tend to become aneuploid/polyploid (31–37).

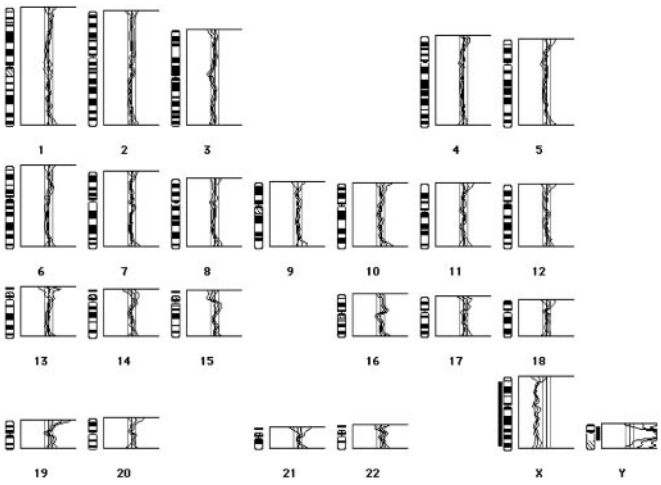

Fig. 2. A representative CGH result of one tumor, 131c, which showed loss of heterozygosity as the second mutational hit at *APC* by microsatellite analysis. Male tumor DNA was cohybridized with female reference DNA onto normal male metaphase spreads. The composite CGH profile shows 95% confidence intervals of the mean values from six metaphase spreads, with threshold values for chromosomal gain and loss of 1.15 and 0.85, respectively. Gains are indicated by bars to the right, losses by bars to the left, of the chromosomal ideograms. Tumor 131c shows no chromosomal imbalances, with the exception of the sex-mismatch control, a relative gain of the Y and loss of the X chromosome.

Like Shih *et al.* (25), we found LOH at sites on chromosomes 1p and 15q in a minority of colorectal adenomas, although LOH occurred at a lower frequency in our sample (10% vs. 5% at 1p; 28% vs. 4% at 15q) and was not found on chromosome 18q (28% vs. 0%). It is unlikely that this difference is due to an increased amount of contaminating normal tissue, because 27 of our 49 tumors had previously been shown to have LOH at the *APC* locus. The probable explanation is partly chance, but also that the two studies used different methods (with different specificity and sensitivity) and analyzed tumors of different origin (from sporadic cases and FAP patients, respectively). We disagree with the view of Shih *et al.* (25) that the LOH results indicate that karyotypic instability is common in early colorectal adenomas. First, we found three only cases of aneuploidy/polyploidy in 20 tumors analyzed by FCM and CGH analysis. Second, three polyps with detectable LOH were normal by CGH analysis. Third, we found that LOH at *APC* did not result from physical loss of material but probably from somatic recombination, inconsistent with Shih *et al.*'s (25) view that CIN precedes *APC* inactivation.

Our results do not support the hypothesis that *APC* mutations are selected for effects on chromosomal mis-segregation manifesting as karyotypic instability in early stages of colorectal tumorigenesis, although we cannot exclude a minor tendency to CIN. It is evident, moreover, that at least some near-diploid colorectal carcinomas have two mutational hits at *APC*. Thus, whereas APC may well have a role in interacting with, or perhaps controlling, the mitotic spindle, loss of this C-terminal function does not inevitably lead to spindle dysfunction and genomic instability, even in late lesions in which cell cycle checkpoints are likely to be deranged.

In summary, our data and earlier results (6, 7) [together with the findings of Haigis *et al.* (30)] show that *APC* mutations are common in colorectal tumors because they provide cells with a direct selective advantage. The nature of that advantage remains largely unknown, but probably primarily involves changes in β-catenin levels. The APC protein may physically associate with components of the mitotic spindle, but its role,

if any, in chromosomal segregation is not yet characterized. The model cell systems previously used to study the association of APC with chromosomal mis-segregation are themselves prone to spontaneous changes in chromosome number and structure, even in the presence of wild-type APC (38, 39). We cannot yet be certain whether or not *APC* mutations increase the tendency for chromosomal mis-segregation to occur in human tumors *in vivo*, but, if mutant APC does have this effect, its consequences do not generally manifest until later-stage tumorigenesis.

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1. Sieber, O. M., Tomlinson, I. P. & Lamlum, H. (2000) *Mol. Med. Today* **6,** 462–469.
2. Fearnhead, N. S., Britton, M. P. & Bodmer, W. F. (2001) *Hum. Mol. Genet.* **10,** 721–733.
3. Miyoshi, Y., Nagase, H., Ando, H., Horii, A., Ichii, S., Nakatsuru, S., Aoki, T., Miki, Y., Mori, T. & Nakamura, Y. (1992) *Hum. Mol. Genet.* **1,** 229–233.
4. Ichii, S., Takeda, S., Horii, A., Nakatsuru, S., Miyoshi, Y., Emi, M., Fujiwara, Y., Koyama, K., Furuyama, J., Utsunomiya, J., *et al*. (1993) *Oncogene* **8,** 2399–2405.
5. Cottrell, S., Bicknell, D., Kaklamanis, L. & Bodmer, W. F. (1992) *Lancet* **340,** 626–630.
6. Lamlum, H., Ilyas, M., Rowan, A., Clark, S., Johnson, V., Bell, J., Frayling, I., Efstathiou, J., Pack, K., Payne, S., *et al*. (1999) *Nat. Med.* **5,** 1071–1075.
7. Rowan, A. J., Lamlum, H., Ilyas, M., Wheeler, J., Straub, J., Papadopoulou, A., Bicknell, D., Bodmer, W. F. & Tomlinson, I. P. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 3352–3357.
8. Smith, K. J., Levy, D. B., Maupin, P., Pollard, T. D., Vogelstein, B. & Kinzler, K. W. (1994) *Cancer Res.* **54,** 3672–3675.
9. Munemitsu, S., Souza, B., Muller, O., Albert, I., Rubinfeld, B. & Polakis, P. (1994) *Cancer Res.* **54,** 3676–3681.
10. Deka, J., Kuhlmann, J. & Muller, O. (1998) *Eur. J. Biochem.* **253,** 591–597.
11. Munemitsu, S., Albert, I., Souza, B., Rubinfeld, B. & Polakis, P. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 3046–3050.
12. Rubinfeld, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S. & Polakis, P. (1996) *Science* **272,** 1023–1026.
13. Rubinfeld, B., Albert, I., Porfiri, E., Munemitsu, S. & Polakis, P. (1997) *Cancer Res.* **57,** 4624–4630.
14. Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B. & Clevers, H. (1997) *Science* **275,** 1784–1787.
15. Kishida, S., Yamamoto, H., Ikeda, S., Kishida, M., Sakamoto, I., Koyama, S. & Kikuchi, A. (1998) *J. Biol. Chem.* **273,** 10823–10826.
16. Ilyas, M., Tomlinson, I. P., Rowan, A., Pignatelli, M. & Bodmer, W. F. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 10330–10334.
17. Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. & Kinzler, K. W. (1997) *Science* **275,** 1787–1790.
18. Sparks, A. B., Morin, P. J., Vogelstein, B. & Kinzler, K. W. (1998) *Cancer Res.* **58,** 1130–1134.
19. Samowitz, W. S., Powers, M. D., Spirio, L. N., Nollet, F., van Roy, F. & Slattery, M. L. (1999) *Cancer Res.* **59,** 1442–1444.
20. Nathke, I. S., Adams, C. L., Polakis, P., Sellin, J. H. & Nelson, W. J. (1996) *J. Cell Biol.* **134,** 165–179.
21. Ishidate, T., Matsumine, A., Toyoshima, K. & Akiyama, T. (2000) *Oncogene* **19,** 365–372.
22. Kawasaki, Y., Senda, T., Ishidate, T., Koyama, R., Morishita, T., Iwayama, Y., Higuchi, O. & Akiyama, T. (2000) *Science* **289,** 1194–1197.
23. Fodde, R., Kuipers, J., Rosenberg, C., Smits, R., Kielman, M., Gaspar, C., van Es, J. H., Breukel, C., Wiegant, J., Giles, R. H. & Clevers, H. (2001) *Nat. Cell Biol.* **3,** 433–438.
24. Kaplan, K. B., Burds, A. A., Swedlow, J. R., Bekir, S. S., Sorger, P. K. & Nathke, I. S. (2001) *Nat. Cell Biol.* **3,** 429–432.
25. Shih, I. M., Zhou, W., Goodman, S. N., Lengauer, C., Kinzler, K. W. & Vogelstein, B. (2001) *Cancer Res.* **61,** 818–822.
26. Roylance, R., Gorman, P., Harris, W., Liebmann, R., Barnes, D., Hanby, A. & Sheer, D. (1999) *Cancer Res.* **59,** 1433–1436.
27. Telenius, H., Carter, N. P., Bebb, C. E., Nordenskjold, M., Ponder, B. A. & Tunnacliffe, A. (1992) *Genomics* **13,** 718–725.
28. Lamlum, H., Papadopoulou, A., Ilyas, M., Rowan, A., Gillet, C., Hanby, A., Talbot, I., Bodmer, W. & Tomlinson, I. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 2225–2228.
29. Abdel-Rahman, W. M., Katsura, K., Rens, W., Gorman, P. A., Sheer, D., Bicknell, D., Bodmer, W. F., Arends, M. J., Wyllie, A. H. & Edwards, P. A. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 2538–2543.
30. Haigis, K. M., Caya, J. G., Reichelderfer, M. & Dove, W. F. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 8927–8931.
31. Weiss, H., Wildner, G. P., Jacobasch, K. H., Heinz, U. & Schaelicke, W. (1985) *Oncology* **42,** 33–41.
32. van den Ingh, H. F., Griffioen, G. & Cornelisse, C. J. (1985) *Cancer Res.* **45,** 3392–3397.
33. Quirke, P., Fozard, J. B., Dixon, M. F., Dyson, J. E., Giles, G. R. & Bird, C. C. (1986) *Br. J. Cancer* **53,** 477–481.
34. Goh, H. S. (1987) *Ann. Acad. Med. Singapore* **16,** 535–538.
35. Hamada, S., Namura, K., Itoh, R. & Fujita, S. (1987) *Jpn. J. Cancer Res.* **78,** 826–832.
36. Giaretti, W., Sciallero, S., Bruno, S., Geido, E., Aste, H. & Di Vinci, A. (1988) *Cytometry* **9,** 238–244.
37. Suzuki, S., Mizuno, M., Tomoda, J., Ohmori, M. & Tsuji, T. (1995) *Gastroenterology* **109,** 1098–1104.
38. Longo, L., Bygrave, A., Grosveld, F. G. & Pandolfi, P. P. (1997) *Transgenic Res.* **6,** 321–328.
39. Liu, X., Wu, H., Loring, J., Hormuzdi, S., Disteche, C. M., Bornstein, P. & Jaenisch, R. (1997) *Dev. Dyn.* **209,** 85–91.