

# Germline APC Mutation on the $\beta$ -Catenin Binding Site Is Associated with a Decreased Apoptotic Level in Colorectal Adenomas

Tiziana Venesio, Ph.D., Antonella Balsamo, M.S., Antonella Scordamaglia, M.S., Marta Bertolaso, M.S., Arrigo Arrigoni, M.D., Tatiana Sprujevnik, M.D., Francesco P. Rossini, M.D., Mauro Risio, M.D.

Unit of Pathology, Institute for Cancer Research and Treatment (IRCC) (TV, AB, AS, MB, MR), Candiolo-Torino, Italy; and Unit of Gastroenterology (AA, TS, FPR), San Giovanni Vecchio Hospital, Torino, Italy

Germline mutations in APC tumor suppressor gene are responsible for familial adenomatous polyposis (FAP). A major role of these genetic changes is the constitutive activation of  $\beta$ -catenin-Tcf-4 mediated transcription of nuclear target genes, but other cellular functions can be misregulated. To assess how different APC mutations can drive the early steps of colonic tumorigenesis, we studied the effect of 10 different germline-truncating alterations on the phenotype of the corresponding adenomas. A significant reduction of apoptosis, uncoupled with an increased *c-myc* and cyclin-D1 expression, was seen with a frameshift mutation on codon 1383, in the 20-aa repeats of the  $\beta$ -catenin degradation domain, independent of a somatic alteration on the wild-type allele. The decreased apoptotic level was associated with a higher incidence of cancerization. No other APC mutation was linked with a similar effect, even in presence of a somatic allelic loss. These findings suggest that mutations in critical sites of the  $\beta$ -catenin degradation domain of APC gene can convey a selective advantage to the colonic neoplastic clones by altering the apoptotic surveillance rather than enhancing the  $\beta$ -catenin-Tcf-4 transcription of growth-promoting genes.

**KEY WORDS:** APC, Apoptosis,  $\beta$ -Catenin, Colorectal adenomas, Familial adenomatous polyposis.

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Familial adenomatous polyposis (FAP) is an autosomal-dominant precancerous condition caused by germline alterations in the tumor suppressor gene APC. These mutations are mostly (>98%) frameshift or nonsense mutations leading to a truncated protein (1, 2).

The APC gene product is part of a multiprotein complex that binds and regulates the degradation of cytoplasmic  $\beta$ -catenin (3-5). In the presence of APC mutations,  $\beta$ -catenin escapes degradation and, by binding Tcf-4/LEF-1 factor, activates the transcription of nuclear genes (6-8). *C-myc*, cyclin D1, and *c-jun* were recognized as targets of this complex (9-11). Abnormal levels of these genes directly contribute to neoplastic transformation being involved in the control of the progression of the cell cycle.

The majority of the reported germline APC mutations occur in the first half of the coding region (12) where the 20-amino acid repeats and the conductin/axin-binding SAMP motifs that are involved in  $\beta$ -catenin degradation are located (3). Mutations between codons 1280 and 1500, in the so-called mutation cluster region (MCR), confer a selective advantage to the cells by allowing the acquisition of allelic loss as a "second hit" (13, 14). Although not known for certain, a deranged balance between  $\beta$ -catenin-binding/degradation or through APC dimerization is thought to be the underlying molecular mechanism of this advantage (15). Mouse models (16) and *in vitro* assays (17) have demonstrated consistently that the severity of the disease correlates with the position of the mutations with respect to the  $\beta$ -catenin-binding/degradation repeats.

Alterations of programmed cell death (PCD) can promote colorectal tumorigenesis (18, 19). It was shown that the APC gene plays a role in the PCD control because in colorectal cell lines carrying a mutant form, the wild-type overexpression inhibits cell growth (20). Apoptosis is the morphological end result of PCD. A unique morphological path-

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Address reprint requests to: Mauro Risio, M.D., Unit of Pathology, Institute for Cancer Research and Treatment (IRCC), 10060 Candiolo-Torino, Italy; e-mail: mrisio@mauriziano.it; fax: 39-011-993-3480.

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way of apoptosis was identified in colorectal FAP adenomas. In those lesions, apoptosis is mainly evident in the form of multiple intercytoplasmatic, Feulgen-positive inclusions—the so-called Leuchtenberger bodies—at the base of the adenomatous epithelium (21). *In situ* labeling of nuclear DNA fragments detects an early apoptosis-related phenomenon that is thought to have a longer duration than that of apoptotic morphological changes. However, a preserved apoptotic function, in the presence of impaired DNA fragmentation, can be seen in mouse colon mucosa (22). Moreover, not all the cells with DNA breaks proceed to morphological evidence of apoptosis (23). Both markers should therefore be used to assess thoroughly the level of PCD activation.

To evaluate the contribution of different APC mutants to early colorectal tumorigenesis, we investigated the relationships among the position of 10 different APC germline mutations, the status of the second allele, the immunohistochemical expression of  $\beta$ -catenin and two growth-promoting target genes (*c-myc* and cyclin D1), and the PCD activation in the corresponding colonic neoplasia.

## MATERIALS AND METHODS

### Patients

The study was performed on 10 patients who had been proven to be affected by FAP on the basis of the presence of multiple colonic polyps (>100) and family history. All patients had undergone colonoscopy and endoscopic polypectomy. Blood samples were also collected, together with clinical data (Table 1). No extracolonic lesions were found for any individuals. FAP syndrome was definitely confirmed in these patients and their affected relatives by the presence of an APC germline mutation.

### Histology and Immunohistochemistry

A total of 52 colorectal adenomas from the 10 probands were analyzed. All polyps were <10 mm in size (range, 2–10 mm). Thirty colorectal sporadic adenomas, cross-matched for size, histotype, and grade of dysplasia were used as controls. All the samples were fixed in 10% phosphate-buffered formalin and embedded in paraffin according to standard procedures. Four- $\mu$ m-thick sections were cut and stained with H&E. Adenomas were diagnosed and typed according to the WHO criteria (24). Epithelial dysplasia was graded in low (mild and moderate) and high (severe). Grading and staging of colorectal adenocarcinomas were in accordance with the TNM system (25). Leuchtenberger bodies were identified using Feulgen stain and quantitatively graded as + (occasional bodies in the adenomatous epithelium; range: 1–25 per 100 viable cells), ++ (moderate number of intraepithelial bodies; range: 26–75 per 100 viable cells), or +++ (large number of intraepithelial bodies; >75 per 100 viable cells) (21). Apoptotic cells were identified according to Kerr's criteria (compaction and margination of nuclear chromatin, condensation of the cytoplasm, nuclear fragmentation, convolution of the cellular surface with the development of pedunculated protuberances) (26) and scored, when present, together with Leuchtenberger bodies.  $\beta$ -catenin, *c-myc*, and cyclin D1 immunohistochemistry were determined on paraffin sections using the anti  $\beta$ -catenin goat polyclonal antibody C-18 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), the mouse monoclonal antibody anti-cMyc clone 9E11 (Medac Diagnostika, Hamburg, Germany), and the anti-cyclin D1 rabbit polyclonal antibody H-295 (Santa Cruz Biotechnology, Inc.), respectively. Sections were deparaffinized and rehydrated. Endogenous peroxidase was blocked in hydrogen peroxide 3% for 5 minutes. For  $\beta$ -catenin

**TABLE 1. Pathologic Features of the 10 FAP Patients Carrying Different APC Germline Mutations**

Patient No.	Sex/Age at Diagnosis (y)	Analysed Adenomas, <i>n</i> (Histology + IIC)	Adenoma Size (mm)	Histology	Invasive Carcinoma	Apoptotic Bodies/TUNEL Index <sup>a</sup>	$\beta$ -Catenin Cytoplasmic Expression
1	M/31	7	3–7	Tub./LGD	—	++/3.9 $\pm$ 0.7	—
2	F/44	3	3–4	Tub./LGD	—	++/2.5 $\pm$ 0.1	—
3	F/38	4	2–4	Tub./LGD	—	++/2.7 $\pm$ 0.9	—
4	F/36	6	2–6	Tub./LGD	1 (pT3); age 36 y	+++ /2.8 $\pm$ 0.5	—
5	M/39	3	2–4	Tub./LGD	—	+++ /NA	—
6	M/36	6	3–10	Tub./LGD	—	+++ /4.8 $\pm$ 0.9	—
7	M/19	8	4–5	Tub./LGD	—	+++ /4.6 $\pm$ 0.1	—
8	M/38	3	2–4	Tub./LGD	—	+++ /NA	—
9	F/19	6	2–10	Tub./LGD	—	+++ /4.3 $\pm$ 0.1	—
10	F/20	8	2–10	Tub.-Vill./LGD-HGD	2 (pT1 + pT3); age 20 y	+ /1 $\pm$ 0.2	++

IIC, immunohistochemistry; Tub., tubular; Vill., villous; LGD, low-grade dysplasia; HGD, high-grade dysplasia; +, occasional Leuchtenberger bodies; ++, moderate number of Leuchtenberger bodies; +++, large number of Leuchtenberger bodies; N.A., not available; —, negative  $\beta$ -catenin staining; +, +, positive  $\beta$ -catenin staining.

<sup>a</sup>TUNEL Index was calculated as means  $\pm$  SEM.

staining, sections were microwaved in 10 mM citrate buffer, pH 6.0, at 100°C for 10 minutes. Slides were then incubated for 1 hour with the primary antibody (for all clones, 1:80), washed in TBS, and incubated with biotinylated secondary antibodies (DAKO, Carpinteria, CA), followed by StrepABC complex (DAKO) for 30 minutes. Staining was visualized using diaminobenzidine (Sigma, St. Louis, MO). The slides were then weakly counterstained with hematoxylin, dehydrated with xylene, mounted in Entellan (Merck, Darmstadt, Germany), and examined under a standard light microscope.

The TUNEL technique was carried out with the *In Situ* Cell Death Detection—POD Kit (Roche Diagnostic Corporation, Indianapolis, IN) according to the manufacturer instructions. DNA strand break fragments were identified by labeling free 3'-OH ends with modified nucleotides using terminal deoxynucleotidyl transferase (TdT). Briefly, the slides were digested by proteinase K (0.5 µg/mL in 10 mM Tris-HCl, pH 7.4, for 15 minutes) and incubated in the TUNEL (TdT-mediated d-UTP nick end-labeling) reaction mixture in a humidified chamber for 60 minutes at 37°C. Positive nuclei were then detected by diaminobenzidine solution for 10 minutes at room temperature and weakly counterstained with methyl green. Labeled nuclei were regarded as positive irrespective of their staining intensity. The TUNEL Index (*i.e.*, the percentage of labeled and total nuclei) was separately calculated in each sample. To assess the overall level of PCD activation, all adenomatous crypts were examined independently of their spatial orientation and the compartmental distribution of apoptotic cells along the crypt axis (27). TUNEL Index values were reported (Table 1) as means ± SEM of the adenomas of each patient. Quantitative grading of Leuchtenberger bodies and TUNEL index of sporadic adenomas used as controls were calculated cumulatively (28).

### Mutation Analysis

Blood DNA/RNA were extracted from lymphocytes according to standard procedures with proteinase K and the QUIamp RNA Blood Mini Kit (Quiagen, Hilden, Germany). A preliminary screening was performed by protein truncation test (PTT) using a primer set that divides the entire APC cDNA (GenBank accession number M73548) into five overlapping regions (29, 30). Segments 2–5, covering exon 15, were obtained directly from PCR amplification of genomic DNA (300 ng), whereas Segment 1, consisting of exons 1–14, was obtained from an RT-PCR reaction of mRNA, using the First Strand Synthesis Kit (Roche, Diagnostics Corporation, Indianapolis, IN, USA). Unpurified PCR products (2 µL) were then used as template in a 25 µL for PTT analysis with a

TnT/T7 coupled reticulocyte lysate system (Promega, Madison, WI). For any samples with a band-shift, the corresponding fragment was reamplified by PCR and sequenced in the ALFexpress II Sequencer using the AutoLoad Solid Phase Sequencing kit (Amersham Biosciences, Freiburg, Germany), and the results were analyzed by the ALFwin 2.00 program (Amersham Biosciences).

To date, only a few missense variants have been described on the APC gene, yet these could have been associated with the gain of a new, aggressive phenotype (31). To verify the presence of these variants, the entire APC coding region was subjected to an enzymatic mutation detection test (EMD; Passport Mutation Scanning Kit—Amersham Biosciences, Freiburg, Germany). This assay is based on the recognition and cleavage of mismatch structures in duplex DNA by the bacteriophage resolvase T4 endonuclease VII (32). Overlapping fragments of 600 bp or 1.2 kb, covering the entire APC cDNA, were amplified by PCR and hybridized with the corresponding wild-type probe by denaturation for 5 minutes at 95°C and cooling at room temperature for 5 minutes. Samples were then subjected to the enzymatic cleavage by T4 Endonuclease VII at 37°C for 30 minutes and evaluated in the sequencer by the ALFwin Fragment Analyser (Amersham Biosciences, Freiburg, Germany). The set of primers used for sequencing reactions and EMD analysis on APC cDNA/DNA are reported in Table 2.

### Somatic Analysis of the APC Second Allele

DNA was extracted in 23 randomly selected and microdissected cases from the 52 formalin-fixed, paraffin-embedded adenomas previously histologically and immunohistochemically analyzed by using the Qiagen Tissue Extraction Kit (Qiagen, Hilden, Germany). Two microsatellite markers, D5S346 and D5S656, located about 32 kb and 396 kb at 3' of APC, were chosen from [www.hgmp.mrc.ac.uk](http://www.hgmp.mrc.ac.uk) to detect allelic losses. Genomic DNA purified from blood lymphocytes and paired paraffin-embedded colorectal adenomas was amplified by PCR using Cy5 fluorescently labeled oligonucleotides, in a reaction volume of 25 µL, in standard PCR buffer (Perkin-Elmer, Norwalk, CT). Cycling conditions were 94°C for 4 minutes, followed by 32 cycles of 94°C, 56–50°C, and 72°C for 1 minute each, and a final 7 minutes of incubation at 72°C. Products were detected by the ALF Express II Sequencer, and results were analyzed using the Allele Links 1.00 program. Allelic loss was scored when there was a reduction of ≥50% of the area of one of the allele. APC deletions were also analyzed by polymorphisms on exons 11 and 15N and in the 3'-UTR, detected respectively by

**TABLE 2. Oligonucleotide Primers Used for Sequencing Reactions and EMD Assay of APC Gene from cDNA for Exons 1–14 and Genomic DNA for Exon 15**

Exons/Codons	Oligonucleotide Sequences (5' to 3'), Forward and Reverse
1–14/1–189	GCTGCAGCTTCATATGATC and TGAGATTCTGAAGTTGAGCG
1–14/159–344	CGCTCAACTTCAGAATCTCA and CAGACTGTCCGATGGATATAC
1–14/340–529	CAAGACAGCTGTATATCCATGC and AAGTGCTCTCATGCAGCCTT
1–14/516–709	AAGGCTACGCTATGCTCTATGA and GGTTCTTGAGCATGCTAACTT
15/651–834	CCACAGGCCAAATCCTAAGAG and GGAGCTGGGTAACACTGTAG
15/814–987	TAATACTGGCAACATGACTGT and CTTACTTTCATCATCTTCAG
15/981–1184	CTCGATTGAATCCTATTCTGAAG and CTGTGATGATGAAGGAATATCTGT
15/1183–1370	TGCCACAGATATTCCTTCATC and TTGGGTGTCTGAGCACCACCTT
15/1359–1618	AAATCTCCCTCCAAAAGTGG and AGCATTACTGCAGCTTGCT
15/1470–1992	CCTGTGGCAAGGAAACCAAGT and GGTTCTCCCTGTGAGTCAG
15/1959–2345	CCAGTTTGCTTTTCTCATAATCCT and CTAGGGGATGATGTCCTTGG
15/2321–2714	TACAGTCTCCTGGCCGAAACT and GATTTTCCAAACCCACGGTA
15/2493–2883	TCTCTATCCACACATTCGTCTGTT and CTTCAGAAACAAAACCTCTAA

*RsaI*, *MspI*, and *SspI* restriction enzymes, using the PCR conditions as described at [www.hgmp.mrc.ac.uk](http://www.hgmp.mrc.ac.uk). Somatic retention or loss of the germline mutation was also assessed by direct sequencing of the fragments of interest. The presence of a second truncated alteration on the MCR was screened for by investigating the region between codons 1183 and 1468, using the EMD assay as described above. The entire MCR was amplified in two overlapping fragments using oligonucleotides for codons 1183–1370 and codons 1359–1468 (Table 2). Polymerase chain reactions were performed in a 50- $\mu$ L volume using AmpliTaq (Perkin-Elmer, Norwalk, CT) and 0.2  $\mu$ M oligonucleotides in 35 cycles (94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute). Mutations were identified in heterozygous samples containing up to a 20-fold excess of normal DNA (32).

## RESULTS

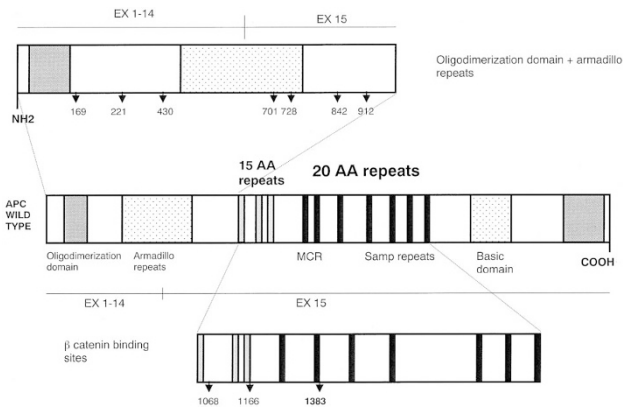
All the found germline APC mutations resulted in truncated proteins, mostly because of a frameshift for the insertions and deletions of a few base pairs (prevalently 1 or 2 bp; Table 3). Although all the mutations occurred in the first 5' half of the coding region, they were scattered throughout this part of the sequence and led to truncations at different domains (Fig. 1). Mutations on codons 169, 221, and 430, located respectively on exons 4, 6, and 9, gave rise to short products preserving only the initial oligodimerization domain (Fig. 1). All the other truncations were found on exon 15, with peculiar differences in respect to the structural motifs of the APC gene (Fig. 1). Actually, chain-terminating mutations on codons 700, 728, 842, and 912 caused the lack of the entire  $\beta$ -catenin-binding/degradation domain. Among these, MT-842 and MT-912 re-

**TABLE 3. APC Germline Mutations and Alterations of the Wild-Type Allele Found in Corresponding Adenomas**

Patient No.	Germline Mutation, Codon (Exon No.)	Alteration Type (Nucleotide Change)	Adenomas Analyzed for the 2nd Hit	Adenomas with Deletion/2nd Truncating Mutations <sup>a</sup>
1	169 (4)	505del4 AGAatagAT	3	0
2	221 (6)	664 C >T TTcAGC	3	1
3	430 (9)	1289–1290 GGcTCAT	2	0
4	701 (15)	2101–2102 insT ACAtTG	3	0
5	728 (15)	2182 delA AGGaGAT	NA	—
6	842 (15)	2523–2524 insA TTAaGAT	3	1/1
7	912 (15)	2734 delT GAAtTA	3	2
8	1068 (15)	3202 del4 CAAtcaaGG	NA	—
9	1166 (15)	3495–3496 insA AAATaAT	2	0
10	1383 (15)	4147delAT CTCatG	4	1

NA, not available.

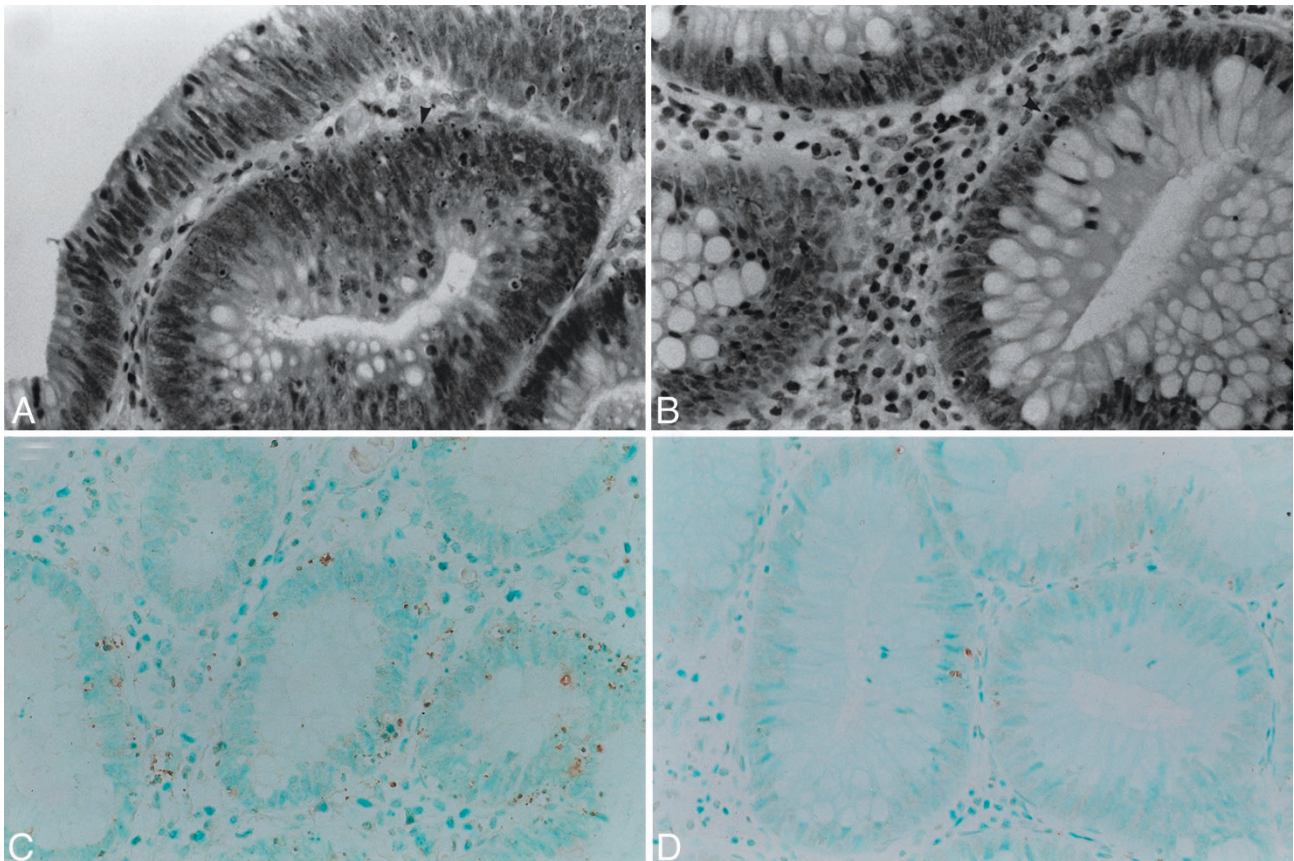
<sup>a</sup> Deletions were detected by microsatellites (D5S346 and D5S656). Only the region encompassing the MCR (between codons 1183 and 1507) was analyzed for the second truncating alterations by the EMD assay.



**FIGURE 1.** Schematic representation of APC gene and the particular position of the FAP mutations found in respect to the main domains of the coding sequence. APC gene is a 2843-aa protein containing an oligodimerization domain, armadillo repeats, three  $\beta$ -catenin-binding regions, seven 20-aa repeats, SAMP repeats, the basic domain, and the EB-1 binding site. Mutations on codons 169, 221, and 430 were localized between the oligodimerization domain and the armadillo repeats region; alterations on codons 701 and 728 mapped on the armadillo repeats region; and lesions on codons 842 and 912 harbored between the armadillo motifs and the  $\beta$ -catenin-binding sites. Chain termination on codon 1068 was situated between the first and second 15-aa repeats; alteration on codon 1166 maps on the third 15-aa repeats; and the frameshift on codon 1383 is localized on the second 20-aa motifs.

tained the complete armadillo repeats. On the  $\beta$ -catenin-binding/degradation region, lesions on codons 1068 and 1166 provided the corresponding proteins with just 1 or 2 of the 15-aa repeats, whereas MT-1383 gave rise to a product that had retained the first 20-aa repeats and lost the SAMP motifs. No alterations other than truncating mutations were found in our samples.

Somatic losses of heterozygosity (LOH) were detected in five adenomas (20% of the cases), with germline truncations respectively on codons 221, 842, 912, and 1383 (Table 4). In the adenomas associated with MT-1383 and MT-912, the allelic losses had occurred between exon 11 and exon 15N, whereas one polyp with MT-842 showed allelic loss for all the three polymorphisms (Table 4; Fig. 3C). In tissue samples, the direct sequencing of the DNA fragments with the germline mutations showed that one case with MT-842 had lost the allele carrying the germline truncation (Table 4; Fig. 3A, B). Adenomas without an evident LOH were then checked for the presence of a second truncating mutation in the MCR (codons 1183–1468). A so-



**FIGURE 2.** Differences in the PCD level correlated with mutations on the  $\beta$ -catenin-binding/degradation sites. **A, B**, adenomas from mutation on codon 912 with the presence of a high number of Leuchtenberger bodies (+++; *arrow*; **A**) and from APC mutation 1383 characterized by few Leuchtenberger bodies (+; *arrow*; **B**); H&E staining, magnification, 40 $\times$ . **C, D**, sections from the same adenomas analyzed by the TUNEL technique, showing high levels of PCD activation for mutation on codon 912 (**C**) and low levels for MT-1383 (**D**); weakly counterstained with methyl green, magnification, 40 $\times$ .

**TABLE 4. APC Gene Deletions Found in Adenomas of FAP Patients Carrying a Characterized APC Germline Mutation**

Patient No.	Germline Mutation, Codon (Exon No.)	Adenomas with Loss ( <i>n</i> )	Germline Mutations in Adenomas <sup>a</sup>	APC Intragenic Polymorphisms in Adenomas <sup>b</sup>		
				Exon 11	Exon 15N	3'-UTR
2	221 (4)	1	Retained	Normal	NI	NI
6	842 (15)	1	Lost	LOH	LOH	LOH
7	912 (15)	2	Retained	Normal	LOH	LOH
			ND	Normal	LOH	LOH
10	1383 (15)	1	Retained	Normal	LOH	LOH

NI, not informative; LOH, loss of heterozygosity; 3'-UTR, 3' untranslated region.

<sup>a</sup>All adenomas with LOH had lost the wild-type allele except one sample from a MT-842 carrier.

<sup>b</sup>The APC somatic allele deletions found by microsatellites were characterized for the intragenic LOH using polymorphisms on exon 11, 15 N, and the 3'-UTR region.

matic truncating mutation was detected in one case associated with MT-842 (Table 3).

Colorectal neoplasia associated with truncation at codon 1383 (Patient 10) showed unique pathologic features in comparison with those observed in the other patients (Table 1). Besides the earlier onset of two adenocarcinomas (age of onset: 20 y; stage pT1 and pT3, respectively) and the prevalence of adenomas with villous architecture, a strongly reduced number of Leuchtenberger bodies was seen in the adenomatous epithelium (Fig. 2). TUNEL Index strictly paralleled the presence of Leuchtenberger bodies in all tested adenomas (Table 1). Consistently, MT-1383 adenomas showed the lowest level of apoptosis and the lowest TUNEL index in the series (Table 1; Fig. 2). The three cases (Patients 1, 2, and 3, associated respectively with MT-169, MT-221, and MT-430) in which the mutations were located between the oligodimerization domain and the c-terminal of the armadillo repeats displayed intermediate levels of both Leuchtenberger bodies and TUNEL index (Table 1 and Fig. 1). Independently of the position of the APC germline mutations, however, the quantity of Leuchtenberger bodies and TUNEL index turned out to be significantly higher in FAP with respect to sporadic adenomas (TUNEL index:  $3.3 \pm 0.4$  versus  $1.91 \pm 0.2$ ;  $P < .05$ ; Table 1).

Cytoplasmic immunostaining for  $\beta$ -catenin was detected only in the adenomas of patients carrying mutation 1383, whereas samples from all other patients showed the weak membrane expression usually seen in the normal epithelium (Table 1; Fig. 4). In no cases were seen  $\beta$ -catenin nuclear staining, *c-myc*, and cyclin D1 immunoreactivity (data not shown).

## DISCUSSION

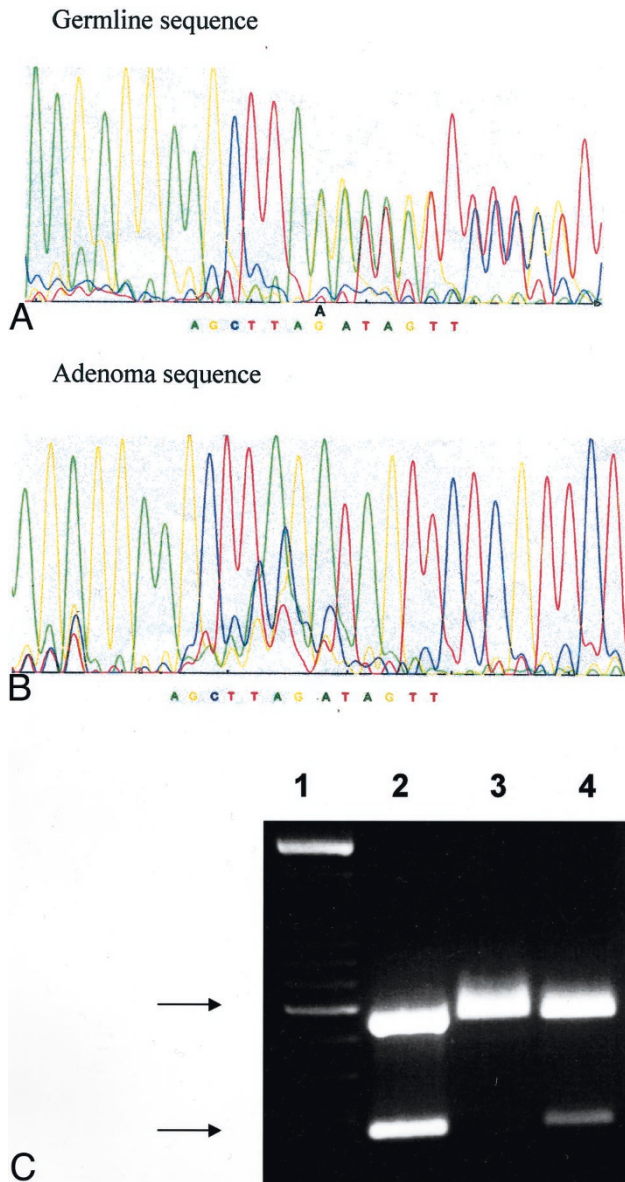
Misregulation of apoptosis can promote colorectal tumorigenesis by two distinct mechanisms. First, it allows the accumulation of proliferating cells in the colonic crypts, it being well known that hyperproliferation coupled with hyperplasia are

early morphogenetic events leading to the development of microadenomas (33, 34). Under this condition, the homeostatic balance between mitotic activity and apoptosis is lost, even if the apoptotic level is higher than in normal mucosa (35). Second, it minimizes phenotypic variations eliminating genetically aberrant cells with enhanced malignant potential from the population (36). In colorectal adenomas, the reduced apoptotic surveillance could promote the malignant transformation, allowing the onset and expansion of clones with invasive phenotype (19). The early onset of two colorectal invasive adenocarcinoma in the patient carrying an APC mutation on codon 1383 is consistent with this interpretation of tumor progression. Moreover, the results of the current study, showing impairment of both morphologically identifiable apoptosis and DNA fragmentation, suggest that the action of nuclear endonuclease is the mechanism activating apoptosis in FAP adenomas.

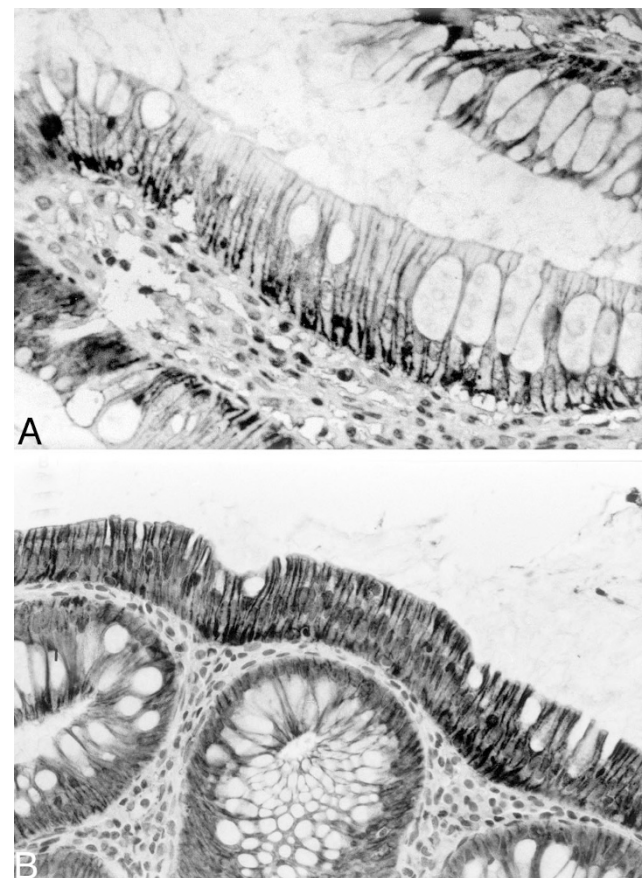
APC gene alterations play a role in the control of cellular homeostasis (20), interfering with apoptotic surveillance. The data presented here indicate that this control can be modulated by the exact position of APC alterations, as only the truncation on codon 1383 displayed a consistent reduction in apoptosis. One of the first hallmarks in programmed cell death is represented by the dismantling of cell-cell contacts. Some of the structural changes involved in this process could be related to the activation of caspases that cleave proteins fundamental for the cell integrity (37). APC was recognized as a substrate of caspase-3, which is able to separate the armadillo repeats from the  $\beta$ -catenin-binding sites (38, 39). Interestingly, the caspase-3 activity leaves the armadillo repeats region of APC protein unaffected, suggesting a possible role of this domain in the apoptotic process. Consistently, in our study, only the truncation of APC protein on codon 1383 displayed significant reduction of apoptosis, whereas the three mutations located between the oligodimerization domain and the c-terminal of the armadillo repeats showed an intermediate level of apoptosis and TUNEL index.

Mutations in APC can stabilize cytoplasmic  $\beta$ -catenin and lead to constitutive LEF/Tcf binding and nuclear signaling (7, 8). An increasing level of  $\beta$ -catenin cytoplasmic and nuclear expression was shown consistently throughout the colorectal tu-

mor progression (40). Although the APC gene plays a pivotal role in the control of  $\beta$ -catenin stability, it was demonstrated that APC mutations alone are not sufficient for the nuclear expression of  $\beta$ -catenin in large, highly proliferating adenomas (41). An altered or overexpressed  $\beta$ -catenin was shown to function as an oncogene preventing cells from suspension-induced apoptosis (anoikis) (42). In our study, adenomatous tissues from MT-1383 carrier showed cytoplasmic without nuclear staining of  $\beta$ -catenin in the absence of *c-myc* and cyclin D1 immunoreactivity. Therefore, we hypothesize that in these tissue samples, the cellular  $\beta$ -catenin turnover already had been altered, with the cytoplasmic accumulation acting as  $\beta$ -catenin overexpression. Nevertheless, in the same tissue samples, the stabilized  $\beta$ -catenin was unable to translocate into the nucleus and transactivate target genes as shown by the lack of nuclear  $\beta$ -catenin, *c-myc*, and cyclin D1 immunostaining. Taken together, these data indicate that in the early phase of colon cancer progression, the APC gene alteration on codon 1383



**FIGURE 3.** Analysis of APC second allele in adenomas. Loss of the mutant allele in an adenoma from a patient carrying mutation on codon 842. **A**, DNA sequence analysis of the region of exon 15 that contains the germline mutation 2523–2524 insA from lymphocytes. **B**, from adenomatous cells of the same patient. The bottom chromatograph shows only the remaining normal sequence because the mutant allele has been lost. **C**, allelic loss detected by polymorphism on exon 15N in two adenomas associated with MT-1383. **Lane 1**, DNA 100-bp molecular weight marker with band sizes of 300 bp and 600 bp indicated by arrows. **Lane 2**, germline DNA sample carrying MT-1383. **Lanes 3 and 4**, DNA samples from two different paraffin-embedded adenomas associated to MT-1383. All samples were amplified, digested by *SspI*, and run on a 2% agarose gel. The sample in **Lane 3** shows a marked LOH, whereas the sample in **Lane 4** displays an allelic retention. The greater efficiency of amplification from lymphocyte DNA in respect to DNA extracted from paraffin-embedded adenomas results in a shift and in more intensity of the two bands of the germ-line sample.



**FIGURE 4.** Immunohistochemical expression of  $\beta$ -catenin in adenomas carrying APC germline mutations on the  $\beta$ -catenin-binding/degradation domain. Adenomas with mutations on codon 1166 (**A**) with a  $\beta$ -catenin immunoreactivity confined to cell membrane and adenomas carrying mutation on codon 1383 (**B**) characterized by a diffuse cytoplasmic immunoreactivity for  $\beta$ -catenin; weakly counterstained with H&E, magnification, 25 $\times$ .

decreases the apoptotic control via  $\beta$ -catenin without affecting the contemporaneous expression of cell growth-promoting genes. As a consequence, we suggest that  $\beta$ -catenin cytoplasmic accumulation affects apoptosis independently of the *c-myc* or cyclin D1 gene transactivation.

The morphologically normal enterocytes from the Min/+ mouse are characterized by the presence of truncated form of APC protein together with the full-length product of the wild-type allele. Studies performed on adenomas from these mice evidenced that cells acquire the hyperproliferative phenotype only when the second APC allele is lost (43). We identified a second hit in 25% of examined FAP adenomas. In no case did we detect immunoreactivity for *c-myc* and cyclin D1, demonstrating that the occurrence of the second somatic alteration of APC gene can be uncoupled with the up-regulation of these two target genes. This demonstrates that in colonic cells, the absence of APC wild-type product is not sufficient to achieve an unleashed proliferation.

Our data indicate that the decreased apoptosis displayed by all adenomas with MT-1383 was an initiating event preceding the acquisition of a deletion on the wild-type allele. Consistent with our results, the *in vitro* immortalization of murine colonic epithelial cells carrying a mutant APC allele, by introducing a truncated form of  $\beta$ -catenin, showed that the earliest tumor-associated change is the escape from senescence (44). We can argue that mutation on codon 1383 exerts an anti-apoptotic function by a dominant negative effect on the product of the wild-type allele. A previous study (17) showed that the wild-type APC activity is inhibited *in vitro* by truncation at codon 1309. A dominant negative effect was supposed for this alteration because of the ability of the corresponding truncated protein to stably dimerize with the endogenous wild-type APC, which is prevented in its normal functions. Mutation on codon 1383, being highly stable for the binding with  $\beta$ -catenin, can carry out a similar effect on the product of the wild-type allele.

In conclusion, in FAP syndrome, the presence of an APC germline-truncating mutation on 20-aa repeats conveys a more severe expression of the disease in terms of increased risk of malignant transformation of colon adenomas associated with reduction of apoptotic surveillance. The effect of this mutation, precocious and independent of the presence of an alteration on the wild-type allele, is not triggered by the transactivation of growth-promoting target genes. In contrast, germ-line alterations scattered along the 5' half of the APC coding region are not correlated with a detectable increase of  $\beta$ -catenin level and/or significant changes in apoptosis. The knowledge of the func-

tion of the armadillo repeats would probably clear up the question of how APC germline mutations located at the 5' to the  $\beta$ -catenin-binding domain can contribute to initiating colon transformation.

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