

APC Haploinsufficiency, but Not *CTNNB1* or *CDH1* Gene Mutations, Accounts for a Fraction of Familial Adenomatous Polyposis Patients Without *APC* Truncating Mutations

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SUMMARY: Familial adenomatous polyposis (FAP) is an autosomal dominant condition characterized by the development of hundreds to thousands of colorectal adenomatous polyps. In addition to the classic form, there is also attenuated polyposis (attenuated adenomatous polyposis coli; AAPC), which is characterized by a milder phenotype. FAP/AAPC is caused by germline mutations in the adenomatous polyposis coli (*APC*) gene. Very recently, germline mutations in the base-excision repair gene *MYH* have been associated with recessive inheritance of multiple colorectal adenomas in a subset of patients. *APC* pathogenic alterations are mostly (>95%) represented by frameshift or nonsense mutations leading to the synthesis of a truncated protein. We identified 20 *APC* truncating mutation carriers out of 30 FAP/AAPC patients from different Italian kindreds. In the remaining 10 patients, we searched for alterations other than truncating mutations by enzymatic mutation detection, real-time quantitative RT-PCR, and genotyping of polymorphic markers encompassing the *APC* locus. Moreover, to assess whether mutations of genes interacting with *APC* can substitute or act in association with *APC* alterations, we sequenced both *CTNNB1* (β -catenin) and *CDH1* (E-cadherin) genes. No *CTNNB1* or *CDH1* mutations were found. On the contrary, four patients showed a reduced *APC* gene expression compared with healthy subjects. In three of the four cases, genotyping results were compatible with a constitutive allelic deletion. In one case this conclusion was confirmed by haplotype segregation analysis. Our results support the notion that FAP/AAPC can result from *APC* constitutive haploinsufficiency, with gene deletion being a possible cause of reduced gene expression. (*Lab Invest* 2003, 83:1859–1866).

Familial adenomatous polyposis (FAP) (OMIM 175100) is an autosomal dominant condition characterized by the development of hundreds to thousands of colorectal adenomatous polyps, conferring a very high risk of cancer at a young age. In addition to the classic form, there is also attenuated polyposis (attenuated adenomatous polyposis coli; AAPC), which is characterized by the development of fewer than 100 adenomas and delayed age of cancer onset (Friedl et al, 1996). Colorectal adenomas are the hallmark of FAP syndrome. However, in addition to polyps, affected individuals may also develop different clinical features, such as retinal lesions, desmoid tumors, and mandibular osteomas. Overall, patients with FAP show considerable phenotypic heterogeneity

as exemplified by different number of adenomas, age of cancer onset, and extracolonic manifestations (reviewed in Vasen, 2000).

FAP is caused by germline mutations in the adenomatous polyposis coli (*APC*) tumor suppressor gene, localized on chromosome 5q21-22 (Grodin et al, 1991; Kinzler et al, 1991). Pathogenic alterations are mostly (>95%) represented by frameshift or nonsense mutations leading to the synthesis of a truncated protein (Laurent-Puig et al, 1998; Powell et al, 1993). Germline alterations are usually detected in 70% of classic patients with FAP and in 10% of patients with AAPC (Heinimann et al, 2001; Lamlum et al, 2000; Spirio et al, 1993; van der Luijt et al, 1996b). A genotype-phenotype correlation has been observed by several authors, suggesting that clinical variability partially depends on the position of mutations along the gene (reviewed in Fodde and Khan, 1995; Pilarski et al, 1999; Spirio et al, 1993; Varesco et al, 1994; van der Luijt et al, 1995).

Increasing evidence indicates that routinely used PCR-based molecular methods fail to detect *APC* mutations in 5% to 10% of patients and are inadequate for finding gene alterations such as large dele-

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tions or transcription defects (Laken et al, 1999; Mandl et al, 1996). There are a minority of patients who do not show detectable *APC* mutations even after intensive gene screening (Laken et al, 1999), suggesting the existence of additional susceptibility genes. Very recently, germline mutations in the base-excision-repair gene *MYH* have been associated with recessive inheritance of multiple colorectal adenomas in a subset of patients (Al-Tassan et al, 2002; Jones et al, 2002; Sieber et al, 2003).

APC has a gatekeeper role in colonic homeostasis. The protein plays a role in the Wnt signaling pathway by catalyzing the degradation of β -catenin (Munemitsu et al, 1995; Rubinfeld et al, 1996). As a consequence of *APC* inactivation, β -catenin accumulates in the cytoplasm and moves to the nucleus, where it mediates the transcription of Wnt target genes (He et al, 1998; Tetsu and McCormick, 1999). β -catenin also plays a role in cell-cell adhesion by interacting with E-cadherin at the adherens junctions (Orsulic et al, 1999). Down-regulation of E-cadherin can be expected to shift β -catenin from adherens junctions to the cytoplasmic pool. However, no E-cadherin mutations have been demonstrated to directly increase β -catenin-dependent transcription of Wnt target genes (Suriano et al, 2003; van de Wetering et al, 2001; Wong and Gumbiner, 2003). On the other hand, reduction of E-cadherin level has been shown to result in alterations of epithelial cell proliferation, migration, and apoptosis (Perry et al, 1999; Smits et al, 2000).

In this study we thoroughly investigated FAP/AAPC patients who tested negative for *APC* truncating mutations. We used different approaches to search for *APC* alterations other than chain-terminating mutations and to assess whether either β -catenin (*CTNNB1*) or E-cadherin (*CDH1*) gene mutations can substitute (or act in association with) *APC* alterations.

Results

Thirty individuals from different families proven to be affected by classic FAP (22 cases) or by AAPC (8 cases) were screened for *APC*-truncating mutations

by protein truncation test (PTT). We identified 20 mutation carriers: 18 with classic polyposis (18/22: 80%) and 2 with AAPC (2/8: 25%) ($p < 0.01$ by Fisher's exact test). Phenotype heterogeneity was observed among mutation carriers (data not shown) as well as among patients without truncating mutations (clinical and pathologic features are reported in Table 1).

The 10 patients without apparent mutation, including 6 cases of AAPC and 4 of classic FAP, were further investigated for germline mutations in β -catenin (*CTNNB1*) and E-cadherin (*CDH1*) genes, as well as for *APC* gene alterations other than protein-truncating mutations. Regarding *CTNNB1*, we first sequenced the 5' end (500 bp) of cDNA, where the majority of mutations occurring in sporadic colorectal cancer have been found (Samowitz et al, 1999). The remaining part of cDNA was divided into four fragments that were analyzed by the enzymatic mutation detection (EMD) assay. The *CDH1* gene was investigated by directly sequencing all exons, intron-exon boundaries, and the proximal promoter region. No *CTNNB1* or *CDH1* gene mutations were found.

To further investigate *APC*, we sequenced 500 bp at the 5' end of the cDNA. This was done to ensure that truncating mutations at the very 5' end of the coding region were not missed because of the running conditions we had used for PTT. We also searched for missense mutations along the entire coding region by means of the EMD assay. Neither missense nor truncating mutations at the very 5' end of the gene were found. To assess *APC* transcription level in mutation-negative patients, we performed a real-time quantitative RT-PCR analysis. The 10 samples showed a relative quantity of *APC* cDNA ranging from 0.52 to 1.4. Samples expressing one only allele were expected to give values close to 0.5. Accordingly, we considered patients who showed values from 0.52 to 0.64 (ie, Patients 1 to 4 in Table 2) as possible carriers of a nonfunctional allele.

To investigate whether a relatively lower mRNA level was compatible with a constitutive *APC* gene deletion,

Table 1. Clinical Characteristics of FAP/AAPC Patients Without *APC* Truncating Mutations

Patient no.	Sex/age at diagnosis	Number of colon polyps	Histotype	Size of adenomas (mm)	Presence of carcinomas/extracolonic manifestations	Family history	Diagnosis
1	M/39	>1000	Tub—Vill/LGD	4–8	No/osteomas	Yes	FAP
2	F/29	>1000	Tub/LGD	5–7	No/no	Yes	FAP
3	F/65	<70	Tub—Vill/LGD-HGD	8–10	Yes/no	?	AAPC
4	M/28	>100	Tub/LGD	2–5	No/no	Yes	FAP
5	F/35	<70	Tub/LGD	ND	No/no	?	AAPC
6	F/58	<70	Tub/LGD	<4	No/no	?	AAPC
7	M/16	<70 ^a	Tub/LGD	ND	No/no	Yes	AAPC
8	F/51	<70	Tub/LGD	5–10	Yes/no	Yes	AAPC
9	M/40	<70	Tub/LGD	ND	No/no	?	AAPC
10	M/29	>100	Tub/LGD	3–6	No/no	Yes	FAP

Tub, tubular; Vill, villous; LGD, low-grade dysplasia; HGD, high-grade dysplasia; ND, not determined.

^aThe father of this 16-year-old patient showed <70 colon polyps at 45 years of age.

Table 2. Real-Time Quantitative RT-PCR and Genotype Results for the 10 Patients Without APC-Truncating Mutations

Patient no.	Diagnosis	Relative quantity of APC cDNA	Genotype						
			Promoter	Ex11	Ex15K	Ex15N	3'-UTR	D5S346	D5S656
1	FAP	0.52	Hom	Hom	Hom	Hom	Hom	Hom	Hom
2	FAP	0.60	Hom	Hom	Hom	Hom	Hom	Het	Het
3	AAPC	0.60	Hom	Hom	Hom	Hom	Hom	Hom	Het
4	FAP	0.64	Het	Hom	ND	Het	Het	ND	ND
5	AAPC	0.80	Het	Hom	Hom	Hom	Hom	Het	Het
6	AAPC	0.82	Hom	Hom	Hom	Hom	Hom	Het	Het
7	AAPC	0.89	Het	Het	ND	Het	Het	Het	Het
8	AAPC	1.07	Het	Het	ND	Hom	Het	Het	Het
9	AAPC	1.25	Het	Het	ND	Hom	Het	ND	ND
10	FAP	1.40	Het	Het	ND	Het	Het	Het	Hom

Hom, homozygous; Het, heterozygous; ND, not determined.

patients were genotyped for polymorphic markers mapping at the APC locus. We analyzed five intragenic single nucleotide polymorphisms (SNPs), from the promoter to the 3'-untranslated region (UTR), as well as two polymorphic microsatellites at the 3' of the gene. All subjects retained heterozygosity at one or more intragenic markers, with the exception of four patients (Patients 1, 2, 3, and 6 in Table 2) who proved to be homozygous (suggesting hemizygosity) for all intragenic markers. Taking into account both quantitative RT-PCR and genotyping results, our findings suggest a constitutive APC allelic deletion causing haploinsufficiency in three patients (Patients 1, 2, and 3 in Table 2). For one FAP patient (Patient 1 in Table 2), we could confirm this hypothesis by family investigation: the proband and two affected nieces were apparently homozygous for all tested markers and were carriers of three different haplotypes (Fig. 1). Haplotype results can be explained by assuming hemizygosity at markers spanning the APC locus, consistent with a whole gene deletion cosegregating with FAP disease in the family. In other words, the three affected subjects do not share any haplotype, being carriers of the same allelic deletion encompassing the investigated markers.

Discussion

In this study we thoroughly investigated FAP/AAPC patients who tested negative for APC-truncating mutations by searching for APC alterations other than protein truncations and by analyzing both CTNNB1 and CDH1 genes.

Because of their function and the relatively high mutation frequency in sporadic gastrointestinal tumors (Becker et al, 1994; Samowitz et al, 1999), the CTNNB1 and CDH1 genes can be regarded as possible candidates in predisposition to colorectal cancer. Most of the previous studies on CTNNB1 in FAP patients have been performed by analyzing only exon 3 (Cao et al, 1999; Muller et al, 1998), where the majority of somatic mutations have been found to occur in colorectal cancer (Samowitz et al, 1999). However, it is conceivable that these mutations,

deeply impairing β -catenin function, are not tolerated at the constitutive level. We extended the analysis to the entire CTNNB1 gene, including sites coding for competitive binding of APC, E-cadherin, and TCF4/LEF-1 to β -catenin. However, similarly to other authors (Dobbie and Muller, 1999), we could not find any mutation. The same result was obtained for the CDH1 gene. To the best of our knowledge, this is the first analysis of the gene in FAP/AAPC kindreds. Up to now, the strongest evidence to support a causal role for E-cadherin alterations in cancer predisposition is still the finding that CDH1 mutations are linked to the hereditary diffuse gastric cancer syndrome (Guilford et al, 1998). Moreover, Smits et al (2000) have demonstrated that constitutive Apc and E-cadherin gene mutations are synergistic in intestinal tumor initiation in mice, indicating that E-cadherin mutations enhance APC-driven tumors without affecting tumor progression. In FAP/AAPC patients with no detectable APC-truncating mutations, we also analyzed APC by both EMD and real-time quantitative RT-PCR methods. We observed deficiencies in gene expression in 4 of 10 subjects. Our results on polymorphic markers encompassing the APC locus were compatible with a constitutive allelic deletion in three of the four subjects. Whole gene deletion was supported by pedigree and haplotype analysis in one of these patients, although not proven at the molecular level.

We also found one patient with low APC gene expression who retains heterozygosity at the intragenic markers. This patient is likely to be carrier of a mutation within the APC locus. However, the precise nature of this APC defect may be difficult to define because Yan et al (2002) reported that in one FAP patient with a reduced expression of APC, the entire coding region, as well as the 3'-UTR and promoter, were completely normal.

Sieber et al (2002) have recently developed an assay on genomic DNA to detect APC deletions. With this approach, 7 (12%) of 60 classic FAP and 0 of 143 AAPC patients without apparent APC mutations proved to carry a full gene deletion. These patients were from different European origin. No deletions

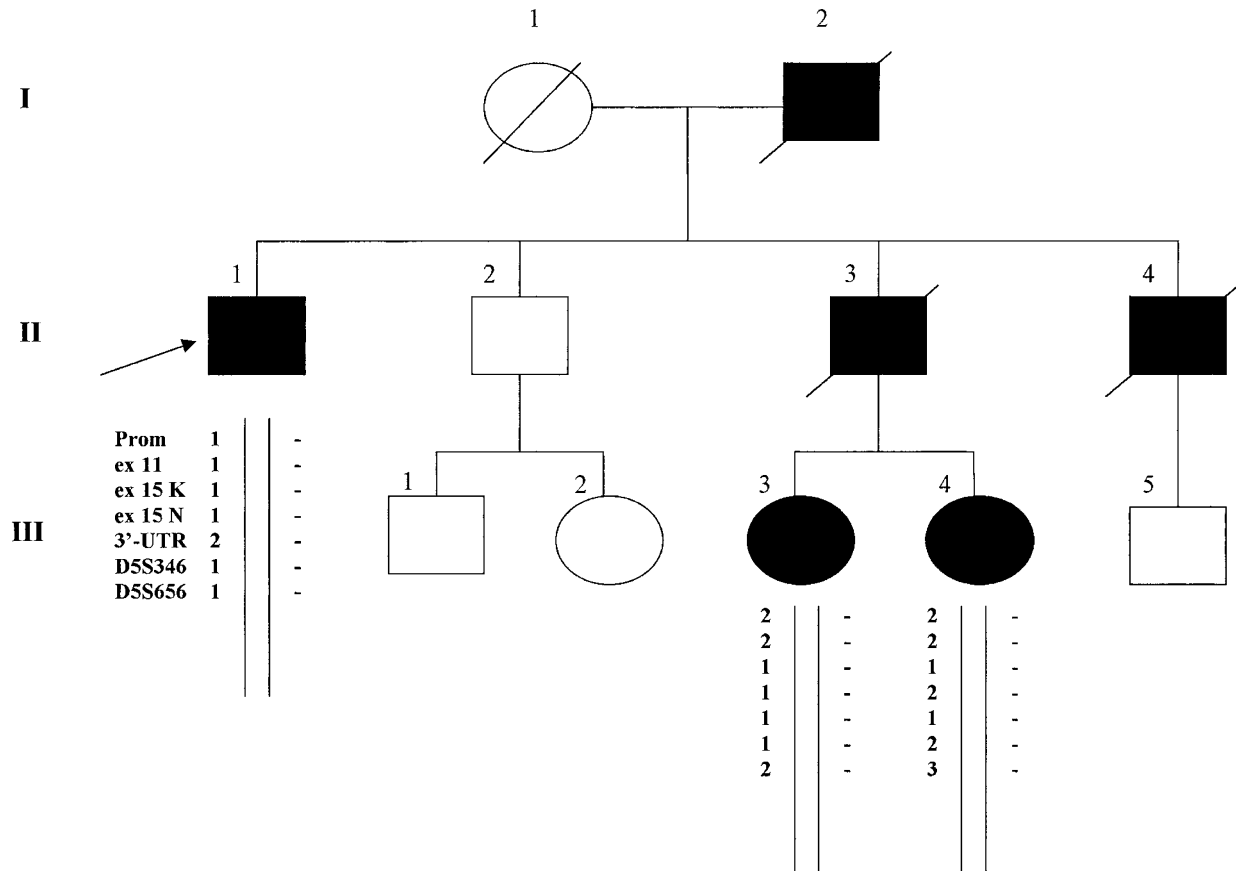


Figure 1.

Pedigree and haplotype analysis of Patient No. 1 (arrow). Filled symbols = clinically affected familial adenomatous polyposis individuals. Five intragenic markers, encompassing *APC* from the promoter to the 3'-UTR region, as well as two microsatellites at the 3' of the gene, have been investigated. The deleted polymorphic alleles are marked by a dash.

were detected in 18 *APC* mutation-negative FAP kindreds from Finland (Moisio et al, 2002). On the contrary, De Rosa et al (1999) reported gene deletions in three (33%) of nine mutation-negative FAP families of Italian origin. Our study on Italian patients suggested gene deletion in 3 of 10 cases. Further studies are needed to more precisely evaluate *APC* deletion frequency in relation to the origin of patients.

Our panel of mutation negative cases also includes six subjects with normal (or slightly reduced) *APC* gene expression. Despite the mutation strategy we used, we could have missed gene alterations in these cases (20% of all tested cases). Moisio et al (2002), by using a comprehensive mutation detection strategy, could not identify *APC* alterations in 28% of Finnish FAP kindreds, including deletions and imbalance in allelic expression.

In the light of the most recent findings (Al-Tassan et al, 2002; Jones et al, 2002; Sieber et al, 2003), mutation assessment of the *MYH* base-excision-repair gene in *APC* alteration-negative patients might represent a relevant issue.

In conclusion, we have shown that constitutive *APC*-reduced expression is present in a fraction of polyposis patients (4/10) with no detectable truncating mutations within the *APC* coding region. However, 6 of 30 patients with a clinical diagnosis of polyposis did

not show evidence of *APC* mutation after intensive molecular screening. We can exclude that mutations of the *CTNNB1* and *CDH1* genes play a significant role in FAP/AAPC predisposition.

Materials and Methods

Patients

Blood samples were obtained from 30 unrelated patients of Italian origin who were diagnosed with FAP or AAPC on the basis of family history and/or endoscopic assessment of colonic polyps. Adenomas were diagnosed and typed according to the World Health Organization criteria (Jass et al, 1990). Overall, 22 patients displayed more than 100 colorectal adenomas (classic FAP) and 8 patients displayed less than 100 adenomas (AAPC). The mean age at polyposis diagnosis was 31 (range, 16–42) years for classic FAP and 40 (range, 16–65) years for AAPC. All patients showed polyps throughout the entire colon; only one patient had extracolonic manifestations (osteomas). After informed consent, blood samples were obtained from one affected individual per family (from three affected subjects in one case) and used for DNA and RNA extraction. DNA and RNA were extracted from lymphocytes by using standard procedure and the QIamp RNA Blood Mini Kit (Qiagen), respectively.

APC gene analysis

PTT. Truncating mutations were investigated by PTT. PTT was performed using a set of primers that allow amplification of the entire cDNA (<http://www.ncbi.nlm.nih.gov/genebank/>; Accession Number M73548) into five overlapping fragments (Bala et al, 1996; van der Luijt et al, 1996a). Fragments 2 to 5 encompassing exon 15 were obtained by directly amplifying genomic DNA by means of *Taq* Extender (Stratagene). Fragment 1, encompassing exons 1 to 14, was obtained by RT-PCR using hexamer primers according to the First Strand Synthesis Kit procedure (Roche). PCR products were used for PTT. The PTT was performed using a TnT/T7 coupled reticulocyte lysate system (Promega). Translation products were loaded on a 14% Tris-glycine-SDS polyacrylamide gel (Bio-Rad). Samples showing a bandshift were reamplified by PCR and sequenced on an ALFexpress II Sequencer (Amersham Biosciences) by using the AutoLoad Solid Phase Sequencing kit (Amersham Biosciences). Results were analyzed by the ALFwin 2.00 program (Amersham Biosciences).

EMD. Base changes were investigated by the Passport Mutation Scanning Kit (Amersham Biosciences), an EMD system. This method, based on the recognition and cleavage of mismatches in duplex DNA

molecules by T4 Endonuclease VII, allows mutation detection with approximately 100% sensitivity and 94% specificity compared with the DNA direct sequencing method (Del Tito et al, 1998). Thirteen overlapping fragments (600–1200 bp) covering the entire *APC* coding region were amplified by PCR using Cy5 fluorescent-labeled primers (Table 3). Following the manufacturer's instructions, each fragment was hybridized with the corresponding wild-type sequence DNA and subjected to enzymatic cleavage by T4 Endonuclease VII. Cleavage fragments were analyzed on an ALFexpress II Sequencer by the ALFwin Fragment Analyser 1.0 Program (Amersham Biosciences).

Genotype Analysis. Genotypes were determined at seven different polymorphic markers encompassing the *APC* locus. The following five markers were SNPs intragenic to *APC*: rs 2019720, located in the promoter region; APC 1458, located within exon 11; APC 5037, located within exon 15K; FB54D, located within exon 15N; and 37A/37B, located within the 3'-UTR. Patients were also genotyped for two microsatellite markers, D5S346 and D5S656, located at 32 kb and 628 kb 3' of *APC*, respectively. All selected SNPs were characterized by allele frequencies close to 0.50. We amplified the polymorphic regions by using primers and PCR conditions as reported at the Genome Database

Table 3. Primers and PCR Conditions

APC and CTNNB1 primers and PCR conditions		
Exons/codons	Forward and reverse (5' > 3')	Annealing temperature (°C)/amplimer size (bp)
APC		
1-14/1-189	GCTGCAGCTTCATATGATC and TGAGATTCTGAAGTTGAGCG	56/581
1-14/159-344	CGCTCAACTTCAGAATCTCA and CAGACTGTGCGATGGATATAC	57/575
1-14/340-529	CAAGACAGCTGTATATCCATGC and AAGTGCTCTCATGCAGCCTT	57/566
1-14/516-709	AAGGCTACGCTATGCTCTATGA and GGTTCTTGAGCATGCTAACTG	58/585
15/651-834	CCACAGGCAAATCCTAAGAG and GGAGCTGGGTAACACTGTAG	58/549
15/814-987	TAATACTGGCAACATGACTGT and CTTACTTTTCATCATCTTCAG	58/519
15/981-1184	CTCGATTGAATCCTATTCTGAAG and CTGTGATGATGAAGGAATATCTGT	58/609
15/1183-1370	TGCCACAGATATTCCTTCATC and TTGGGTGTCTGAGCACCCTTT	60/561
15/1359-1618	AAATCTCCCTCCAAAAGTGG and AGCATTTACTGCAGCTTGCT	58/777
15/1470-1992	CCTGTGGCAAGGAAACCAAGT and GGTTCTCCCTGTGAGTCAG	58/1566
15/1959-2345	CCAGTTTGCTTTTCTCATAATTCCT and CTAGGGGATGATGTCCTTGG	58/1158
15/2321-2714	TACAGTCTCCTGGCCGAAACT and GATTTTCCAAACCCACGGTA	60/1179
15/2493-2883	TCTCTATCCACACATTCGTCTGTT and CTTCCAGAACAAAACCCTCTAA	69/1170
CTNNB1		
1-4/1-126	AAGCCTCTCGGTCTGTGG and TGATGGTTCAGCCAAACGCT	60/600
4-7/112-316	CTGCTCATCATCCCACTAATGTC and TCCACCACTAGCCAGTATGA	56/610
6-10/307-511	AGCAAGCTCATCATACTGGC and CGAATCAATCCAACAGTAGC	56/614
9-14/497-703	ATCCCACTGGCCTCTGATAA and TCATCCTGGCGATATCCAAG	56/617
13-16/688-885	GACTGCTGATCTTGACTTG and GAGCTACTTCAAAGCAAGCAA	56/611
APC oligonucleotides for real-time quantitative RT-PCR		
Oligonucleotides	Sequences (5' > 3')	Amplimer size (bp)
Probe	ATTAGAGCGTCTTAAAGAGCTT	88
Forward primer	TTCCATAAGAACGGAGGGACA	
Reverse primer	TTTGACCGCAGTTTTTACTCCAGGGAAAATTACT	

(<http://www.gdb.org>) and at the NCBI-Single Nucleotide Polymorphism (<http://www.ncbi.nlm.nih.gov/SNP>) web sites. SNPs were investigated by restriction enzyme digestion of PCR products: *RsaI* was used for markers rs 2019720 and APC 1458, *MspI* for FB54D, and *SspI* for T/C 3'-UTR. APC 5037 was investigated by direct sequencing.

Real-Time Quantitative RT-PCR. *APC* expression was assessed by monitoring the mRNA level. We used an assay based on the 5' exonuclease activity of *Taq* DNA polymerase (Gibson et al, 1996; Holland et al, 1991). In addition to the standard PCR components, this assay requires an oligonucleotide probe specific to a sequence between two primers. The probe is labeled with two different fluorophores, a reporter dye at the 5' end and a quencher dye at the 3' end. Fluorescence emission from the reporter is absorbed by the quencher. During the extension phase of each PCR cycle, *Taq* DNA polymerase cleaves the probe annealed between the two primers. The cleavage, by separating the two dyes, enables detection of the reporter fluorescence. The resulting increase in reporter emission is directly proportional to the amount of product generated by each PCR cycle. We obtained *APC* cDNA by RT-PCR as described above. A cDNA fragment of 88 bp, covering the exon 2 to 3 juxtaposition, was used as a target for the assay (Table 3). A cDNA fragment from the *GAPDH* gene was used as an internal control (Applied Biosystems). *APC* and *GAPDH* probes were labeled at the 5' ends with the reporter dyes FAM and JOE, respectively, and at the 3' ends with the TAMRA quencher dye (Applied Biosystem). Both the *APC* primers and probe were designed by using PRIMER EXPRESS software (Applied Biosystems). Probe and primer sequences are listed in Table 3. Real-time quantitative RT-PCR was performed in 25 μ l containing 1 \times *Taq* Man Universal Master mix (Applied Biosystems). The optimal *APC* amplification conditions were as follows: 200 nM for the probe in the presence of 900 nM forward and 150 nM reverse primers. The optimal *GAPDH* amplification conditions were those suggested by the manufacturer (Applied Biosystems). Amplification reactions were performed for 2 minutes at 50 $^{\circ}$ C and 10 minutes at 95 $^{\circ}$ C, followed by 40 cycles of denaturation at 95 $^{\circ}$ C for 15 seconds and annealing at 60 $^{\circ}$ C for 1 minute. We performed two independent experiments. Each experiment comprised (in triplicate) control samples from 2 healthy individuals, samples from the 10 patients with undetectable *APC* mutations, and 2 no-template controls. The reporter emission was measured by an ABI PRISM 7700 Sequence Detection System (Applied Biosystems): the signal is normalized to an internal reference and the software sets the threshold cycle (C_T) when the reference becomes equal to 10 standard deviations of the baseline. We quantified the target *APC* cDNA by calculating the $2^{-(\Delta\Delta C_T)}$ value where $\Delta\Delta C_T = \Delta C_{T1} (C_T \text{ APC} - C_T \text{ GAPDH}) - \Delta C_{T2} (C_T \text{ Calibrator} - C_T \text{ GAPDH})$. We used as a calibrator the sample with the lower C_T value. We normalized the $2^{-(\Delta\Delta C_T)}$ mean value of each patient sample against the $2^{-(\Delta\Delta C_T)}$ mean value of the healthy control sub-

jects. By this approach, samples normally expressing both alleles are expected to give values close to 1, whereas samples expressing only one allele are expected to give values close to 0.5. Table 2 shows mean values from the two independent experiments.

β -Catenin Gene (CTNNB1) Mutation Analysis

The entire β -catenin cDNA was amplified into five fragments of 600 bp each. Primers are reported in Table 3. The PCR fragment corresponding to the 5' terminal region, including the complete GSK3 β phosphorylation consensus motif, was analyzed by direct sequencing as described above. The other four PCR products, encompassing the 12 Armadillo repeats, were subjected to EMD as above.

E-Cadherin Gene (CDH1) Mutation Analysis

The 16 exons and part of the promoter region of the gene were amplified by PCR using the primers and reaction conditions reported by Berx et al (1995). PCR products were sequenced. Sequencing reactions were performed in forward and reverse by using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE) and analyzed on an ABI Prism 310 sequencer (PE).

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