Whole-gene *APC* deletions cause classical familial adenomatous polyposis, but not attenuated polyposis or ''multiple'' colorectal adenomas

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Familial adenomatous polyposis (FAP) is a dominantly inherited colorectal tumor predisposition that results from germ-line mutations in the *APC* gene (chromosome 5q21). FAP shows substantial phenotypic variability: classical polyposis patients develop more than 100 colorectal adenomas, whereas those with attenuated polyposis (AAPC) have fewer than 100 adenomas. A further group of individuals, so-called ''multiple'' adenoma patients, have a phenotype like AAPC, with 3–99 polyps throughout the colorectum, but mostly have no demonstrable germ-line *APC* mutation. Routine mutation detection techniques fail to detect a pathogenic *APC* germ-line mutation in approximately 30% of patients with classical polyposis and 90% of those with AAPC/multiple adenomas. We have developed a real-time quantitative multiplex PCR assay to detect *APC* exon 14 deletions. When this technique was applied to a set of 60 classical polyposis and 143 AAPC/multiple adenoma patients with no apparent *APC* germ-line mutation, deletions were found exclusively in individuals with classical polyposis (7 of 60, 12%). Fine-mapping of the region suggested that the majority (6 of 7) of these deletions encompassed the entire *APC* locus, confirming that haploinsufficiency can result in a classical polyposis phenotype. Screening for germ-line deletions in *APC* mutation-negative individuals with classical polyposis seems warranted.

Familial adenomatous polyposis (FAP) (Mendelian Inheritance in Man 175100) is an autosomal dominant predisposition to hundreds or thousands of colorectal adenomas, which accounts for less than 1% of the total colorectal cancer burden (1). The disorder is caused by germ-line mutations in the adenomatous polyposis coli (*APC*) gene and is classically characterized by more than 100 colorectal adenomas, early onset of colorectal carcinoma, and specific extracolonic features, including congenital hypertrophy of the retinal pigment epithelium, polyposis of the upper gastrointestinal tract, and desmoid tumors (reviewed in ref. 2). In addition to individuals with classical polyposis, there exist attenuated polyposis (AAPC) patients with fewer than 100 adenomas. AAPC patients harbor germ-line mutations in the 5′ and 3′ regions and exon 9 of the *APC* gene (3, 4). A further group of individuals, so-called ''multiple'' adenoma patients, have a phenotype like AAPC, with 3–99 polyps throughout the colorectum, but mostly no demonstrable germ-line *APC* mutation, and often with limited or nonexistent family history of polyposis.

Routine mutation detection techniques [for example, single-strand conformation polymorphism (SSCP) analysis, denaturing gradient gel electrophoresis (DGGE), DNA sequencing, and the protein truncation test (PTT)] identify germ-line *APC* mutations in approximately 70% and 10% of individuals with classical polyposis and AAPC/multiple adenomas, respectively (4–8). The underlying molecular genetic cause(s) therefore remain(s) to be determined in a considerable proportion of apparently *APC* mutation-negative patients. The failure to detect germ-line mutations might also, however, be caused by methodological difficulties, as has recently been shown for another colorectal cancer predisposition, hereditary nonpolyposis colorectal cancer, where over 5% of cases in some populations result from exon-spanning genomic deletions in *hMSH2* (9). By analogy, such submicroscopic deletions may be missed in a substantial fraction of *APC* mutation-negative patients with classical polyposis or AAPC/multiple adenomas. So far, only limited data are available, and no comprehensive studies have been performed to establish the frequency of germ-line *APC* deletions in these patients (10–15).

In this survey, we aimed (*i*) to develop a real-time quantitative multiplex PCR assay (RQM-PCR) to detect *APC* germ-line deletions, (*ii*) to determine their frequency in apparently *APC* mutation-negative patients with classical disease as well as those with AAPC/multiple adenomas, and (*iii*) to fine-map all detected *APC* deletions by using a set of polymorphic markers spanning the entire *APC* gene.

Patients and Methods

Study Population. This study examined 203 unrelated individuals clinically diagnosed with colorectal polyposis and in whom no germ-line *APC* mutation had been identified by using one or more standard mutation detection techniques (SSCP, DGGE, DNA sequencing, PTT). The patients came from diagnostic laboratories in the United Kingdom (Edinburgh, London, Oxford; $n = 167$), Denmark (Copenhagen; $n = 11$), Switzerland

Abbreviations: APC, adenomatous polyposis coli; FAP, familial adenomatous polyposis; AAPC, attenuated adenomatous polyposis coli; RQM-PCR, real-time quantitative multiplex PCR; Alb, albumin.

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Table 1. Primers and probes for the RQM-PCR assay

Primer/probe name	Sequence (5′ to 3′)	Amplicon size, bp
APC exon 14 forward	GCCAGACAAACACTTTAGCCATTA	91
APC exon 14 reverse	TACCTGTGGTCCTCATTTGTAGCTAT	
APC exon 14 probe (5′-FAM, 3′-TAMRA)	CTGGACACATTCCGTAATATCCCACCTCC	
Alb exon 12 forward	TTGCATGAGAAAACGCCAGTA	70
Alb exon 12 reverse	GTCGCCTGTTCACCAAGGA	
Alb exon 12 probe (5′-VIC, 3′-TAMRA)	TCTGTGCAGCATTTGGTGACTCTGTCAC	

(Basel; $n = 11$), Portugal (Lisbon; $n = 9$), and Italy (Naples; $n = 4$). Overall, 60 (30%) patients displayed more than 100 colorectal adenomas (classical polyposis), and 143 (70%) patients displayed between 3 and 99 colorectal adenomas ("multiple adenoma"/AAPC phenotype). Additional phenotypic details (gender, age at diagnosis, family history, extracolonic disease, occurrence of colorectal cancer) were available for 165 (81%) individuals. Written informed consent was obtained from all individuals.

Real-Time Quantitative Multiplex (RQM)-PCR. RQM-PCR determines gene dosage by monitoring PCR amplification in real-time and making use of the 5′ exonuclease activity of *Taq* DNA polymerase. Besides the standard PCR components, this assay requires an excess of an oligonucleotide probe that is specific to a sequence between the two primers. The probe is labeled with two different fluorophores, a reporter dye [6-carboxyfluorescein (6-FAM) or VIC] at the 5′ end and a quencher dye [6-carboxytetramethylrhodamine (TAMRA)] at the 3′ end. While the probe is intact, the emission of the reporter dye is absorbed by the quencher dye. During the extension phase of each PCR cycle, *Taq* DNA polymerase cleaves the oligonucleotide probes annealed between the two primers. Probe cleavage separates the reporter from the quencher dye. The resulting increase in reporter emission can be detected by using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) and is directly proportional to the amount of product being generated each PCR cycle. When probes with different reporter dyes are used, different amplicons can be amplified (multiplexed) in the same reaction.

The primers and probes were designed by using PRIMER EXPRESS software (Applied Biosystems). Exon 14 of *APC* was chosen as target for the assay, as it is encompassed by the majority of submicroscopic *APC* germ-line deletions reported to date (10–13). Exon 12 of human serum albumin (*Alb*), another single-copy gene, was chosen as internal control. *Alb* is located on chromosome 4q11–q13, a region that is not expected to be deleted or amplified in the germ-line of colorectal adenoma patients because it does not show loss of heterozygosity in colorectal adenomas, implicating a germ-line event. All primer and probe sequences were checked for specificity by using the National Center for Biotechnology Information BLAST program (www.ncbi.nlm.nih.gov/blast). The *APC* exon 14 and *Alb* exon 12 probes were labeled at the 5′ ends with the reporter dyes FAM and VIC, respectively, and the 3′ ends with the quencher dye TAMRA (Applied Biosystems). Sequences of the primers and probes are shown in Table 1.

The RQM-PCR assay was optimized by following the instructions in User Bulletin no. 5 (Applied Biosystems). When we used 20 ng of DNA in a 25-μl reaction, the optimal concentrations for *APC* exon 14 and *Alb* exon 12 were 50 nM for both forward primers and 300 nM for both reverse primers. The optimal probe concentrations proved to be 200 nM for *APC* exon 14 and 175 nM for *Alb* exon 12. DNA concentrations between 10 ng and 50 ng were within the linear dynamic range of the system.

Briefly, genomic DNA was isolated from EDTA-containing blood according to the salting-out method (16). DNA aliquots of 15 ng/μl were prepared for RQM-PCR. The assay was carried out in 96-well optical reaction plates sealed with optical adhesive covers (Applied Biosystems). Each experiment comprised in triplicate 3 normal controls, 1 deletion control, 27 patient samples, and 1 no-template control. Each 25-μl reaction contained 1× TaqMan Universal PCR Master mix (Applied Biosystems), 50 nM *APC* exon 14 and *Alb* exon 12 forward primers, 300 nM *APC* exon 14 and *Alb* exon 12 reverse primers, 200 nM *APC* exon 14 probe, 175 nM *Alb* exon 12 probe, and 2 μl of DNA. To reduce the risk of cross-contamination, samples and controls were added after aliquoting the master mix. The plates were centrifuged at 260 × *g* for 1 min, and the reactions were performed on an ABI PRISM 7700 Sequence Detection System. The thermal cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and simultaneous annealing and extension at 60°C for 1 min.

The data obtained were analyzed by using the comparative C_T method (as described in User Bulletin no. 2 and ref. 17). After normalization against the internal *Alb* control, this method allowed determination of *APC* copy number of a given patient by comparison to a normal calibrator. Although the original C_T method relied only on one normal sample as calibrator, we used the mean of three normal samples to obtain a more consistent calibrator value between experiments. The calibrator value (CV) was calculated as follows, with CS denoting the normal calibrator sample: $CV = \{[\Delta C_T\ Alb\ (CS\ 1) - \Delta C_T\ APC\ (CS\ 1)] + [\Delta C_T\ Alb\ (CS\ 2) - \Delta C_T\ APC\ (CS\ 2)] + [\Delta C_T\ Alb\ (CS\ 3) - \Delta C_T\ APC\ (CS\ 3)]\}/3$. *APC* gene copy number was expressed as a $2^{-(\Delta\Delta C_T)}$ value, where $\Delta\Delta C_T = CV - [\Delta C_T\ Alb\ (\text{patient sample}) - \Delta C_T\ APC\ (\text{patient sample})]$. ΔC_T represented the mean C_T value of each sample-triplicate, where C_T was defined as the cycle number during the exponential phase at which the amplification plot passed a fixed normalized emission intensity threshold between 0.03 and 0.13 (average 0.08). Samples without *APC* deletions were expected to give $2^{-(\Delta\Delta C_T)}$ values close to 1, and samples with *APC* deletions were expected to give $2^{-(\Delta\Delta C_T)}$ values close to 0.5. The reliability of the assay was confirmed by using DNA samples of five unrelated, healthy controls and five unrelated FAP patients with previously characterized *APC* deletions encompassing exon 14. The healthy controls displayed $2^{-(\Delta\Delta C_T)}$ values ranging from 0.83 to 0.92, whereas *APC* deletion controls showed $2^{-(\Delta\Delta C_T)}$ values ranging from 0.54 to 0.61 (Fig. 1).

Genotype Analysis. For all classical polyposis patients, genotypes were determined at nine different polymorphic marker loci. The following markers were intragenic to *APC*: (*i*) an A/G polymorphism (National Center for Biotechnology Information single-nucleotide polymorphism cluster ID: rs2019720) located within the promoter region, (*ii*) an A/T polymorphism (rs1914) located within intron 7, (*iii*) a T/C polymorphism located within exon 11 (18, 19), (*iv*) an A/G polymorphism located within exon 15I (20), (*v*) an A/G polymorphism located within exon 15J (19, 21), and

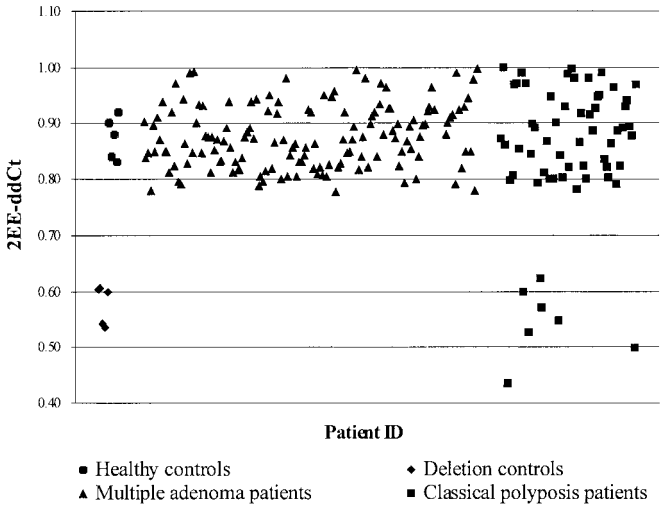

Fig. 1. RQM-PCR results for 5 healthy controls, 5 known deletion controls, 143 AAPC/multiple adenoma patients, and 60 classical polyposis patients. Patients with two copies of *APC* exon 14 display $2^{-(\Delta\Delta CT)}$ values between 0.78 and 1.00, whereas patients with only one copy display values between 0.43 and 0.62 (overall, $2^{-(\Delta\Delta CT)}$ values were consistent with a normal distribution and all *APC* deletion patients displayed $2^{-(\Delta\Delta CT)}$ values >2.9 standard deviations from the mean).

(*vi*) a T/C polymorphism located within the 3′ untranslated region (19, 22). In addition, all patients with identified *APC* deletions were genotyped for two polymorphic markers, an A/G (rs748628) and a C/T polymorphism (rs1922665) located about 110 kb and 37 kb 5′ of *APC*, as well as three microsatellite markers, D5S346, D5S656, and D5S421 located about 32 kb, 396 kb, and 628 kb 3′ of *APC*, respectively (University of California, Santa Cruz Genome Browser, April 1, 2001 freeze at http://genome.ucsc.edu). Allele frequencies for the rs2019720, rs1914, rs748628, and rs1922665 polymorphisms were estimated using DNA from 28 unrelated, healthy European controls. We used an MJ Research Tetrad PCR machine, and the PCR conditions consisted of 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension step at 72°C for 10 min unless specified otherwise in Table 2. Restriction enzyme digestions, as indicated in Table 2, were performed according to the manufacturer's instructions (New England Biolabs). Sequencing reactions were carried out in forward and reverse orientations by using the ABI BigDye Terminator Ready Reaction Mix and analyzed on an ABI 377 semiautomated sequencer. Pyrosequencing was performed using a PSQ 96 System, PSQ 96 SNP Reagent kit, and PSQ 96 Sample Preparation kit (Pyrosequencing AB) according to manufacturer's instructions.

Statistical Analysis. Statistical comparison of index patients' features (gender, age at diagnosis, polyp number, colorectal cancer occurrence, and extracolonic disease) and molecular genetic data (gene dosage, genotype analysis) was performed by using the χ^2 and Fisher's exact tests for categorical variables, or Student's *t* test for continuous variables, with all of the probabilities reported as two-tailed *P*s, considering $P < 0.05$ to be statistically significant.

Results

Analysis of 60 classical polyposis and 143 AAPC/multiple adenoma patients with no apparent *APC* germ-line mutation by using the RQM-PCR assay identified 7 (3.4%) individuals harboring *APC* germ-line deletions encompassing exon 14, with $2^{-(\Delta\Delta CT)}$ values ranging from 0.43 to 0.62 (Fig. 1). Notably, all *APC* deletion patients came from the classical polyposis group, accounting for 11.7% (7 of 60) of these cases, compared with none (0 of 143) in the AAPC/multiple adenoma group ($P < 0.001$, χ^2 test). The phenotypic features of the *APC* deletion patients compared with the remaining *APC* mutation-negative classical polyposis patients were very similar, with comparable

Table 2. Primer sequences, annealing temperatures, detection methods applied, and allele frequencies for the *APC* polymorphisms

APC region	Primer sequence (5′ to 3′)	Annealing temperature, °C	Detection	Allele 1 (allele frequency)	Allele 2 (allele frequency)	Ref.
rs748628	CTTTTCTTTTTCTTTTTCt* CTTTTCTTTTTCTTTTTCc* CTTACTACATTCAAGGGGAT	53	ARMS-PCR	A (0.45)	G (0.55)	†
rs1922665	CTTCCCTGTTCTGCCAATCT GCACTGGATGTTCAGAGACG‡ TCTGTTGGTGGTCTCC§	53	Pyrosequencing	C (0.46)	T (0.54)	†
Promoter	TGGGGATGAGAGAAAGAGGAGGA CGCAAAAAGCCACTACCACTG	60	RsaI digest	A (0.45)	G (0.55)	†
Intron 7	CAGGTTTGAGCCATCATGC ATCCAATCCCTAAGCTTGACTG	60	Sequencing	A (0.46)	T (0.54)	†
Exon 11	GATGATTGTCTTTTTCCTCTTGC CTGAGCTATCTTAAGAAATACATG	55	RsaI digest	T (0.48)	C (0.52)	18, 19
Exon 15I	AGTAAATGCTGCAGTTCAGAGG CCGTGGCATATCATCCCCC	56	BsaJ1 digest	A (0.62)	G (0.38)	20
Exon 15J	CCCAGACTGCTTCAAAATTACC GAGCCTCATCTGTACTTCTGC	55	Sequencing	A (0.57)	G (0.43)	19, 21
3′ Untranslated region	GCATTAAGAGTAAAATTCCTCTTAC ATGACCACCAGGTAGGTGTATT	58	SspI digest	T (0.54)	C (0.48)	19, 22

*Forward primers for A and G allele.
†See *Patients and Methods* for details.
‡5′ Biotinylated reverse primer.
§Sequencing primer.

Table 3. Genotype results for seven classical polyposis patients harbouring an *APC* exon-14 deletion as determined by RQM-PCR

ID	367.vi.4	1749-1	236.vi.11	2350	BAN1773	602	JS
rs748628	**het**	**hom**	**het**	**het**	**het**	**hom**	**het**
rs1922665	**hom**	**hom**	**het**	hom	hom	**het**	**het**
APC promoter	**het**	**het**	hom	hom	hom	hom	hom
APC intron 7	hom	**het**	hom	hom	hom	hom	hom
APC exon 11	hom	**het**	hom	hom	hom	hom	hom
APC exon 14	del	del	del	del	del	del	del
APC exon 15I	hom	hom	hom	hom	hom	hom	hom
APC exon 15J	hom	hom	hom	hom	hom	hom	hom
APC 3′ untranslated region	hom	hom	hom	hom	hom	hom	hom
D5S346	hom	**het**	**het**	hom	hom	hom	hom
MCC locus	del?	**not del**	**not del**	del?	del?	del?	del?
D5S656	**het**	**het**	**hom**	hom	hom	hom	**het**
D5S421	**het**	**het**	**het**	hom	**het**	**het**	**hom**

Bold type indicates areas excluded from the respective *APC* deletions. het, heterozygosity; hom, homozygosity (suggesting hemizygosity); del, deletion; *MCC*, mutated in colorectal cancers.

median age at diagnosis (26.5 vs. 30 years) and similar proportions of positive family history (80.0% vs. 85.1%) and extracolonic disease (28.6% vs. 22.6%).

To assess the molecular extent of the *APC* germ-line deletions identified, six polymorphic markers intragenic to *APC* and five polymorphic markers flanking the *APC* gene were typed (Table 3). Six (85.7%) of seven deletion patients were apparently homozygous (suggesting hemizygosity) at markers spanning the coding region of the *APC* gene, consistent with whole-gene deletions. In three of these cases (602, 236.vi.10, and BAN1773), homozygosity at D5S656 suggested that the deletion might also encompass the *MCC* (mutated in colorectal cancers) locus. In at least one case (1749-1), the germ-line deletion did not involve the whole gene. Assessment of the polymorphic markers 5′ and 3′ of *APC* allowed to determine the maximum extent in six (85.7%) of the seven deletion cases and suggested that at least four (57.1%) were restricted to the *APC* gene (Table 3).

We then assessed whether or not our analysis was likely to have failed to detect any large intragenic deletions 5′ or 3′ of exon 14 in 50 of the patients with classical FAP. Thirteen (26.0%) of these patients were homozygous for all intragenic markers. Two (4.0%) additional patients were homozygous for the three intragenic markers 5′ of exon 14. There was no evidence for an over-representation of homozygotes at any of the polymorphisms studied (data not shown). Although we cannot exclude the possibility that our analysis failed to identify a small number of patients with *APC* deletions, our data suggest that we did not miss large numbers of deletions and, therefore, such changes cannot account for all of the *APC* mutation-negative patients with classical FAP.

Discussion

Applying the RQM-PCR assay for the detection of *APC* exon-14 germ-line deletions, we identified 7 deletion patients in a set of 60 classical polyposis and 143 AAPC/multiple adenoma patients with no apparent *APC* germ-line mutation. Although previous studies of germ-line *APC* deletions have not consistently reported classical disease in these patients, all of our deletion patients displayed classical polyposis, exhibiting more than 100 adenomas. In contrast to the multiple adenoma subgroup (3–99 polyps), where no deletion could be detected, the frequency of *APC* deletions in patients with classical polyposis amounted to 11.7% of mutation-negative cases. Fine-mapping of the detected *APC* deletions (including available affected and unaffected relatives) by using six intragenic, polymorphic markers suggested a whole-gene deletion in six of seven patients (85.7%; Table 3).

Our results make an interesting comparison with previous data from AAPC patients. It has been suggested that in some cases, AAPC can result from instability of the mutant APC protein (23). van der Luijt *et al.* (24), for example, reported that truncating germ-line *APC* mutations in the 3′ half of exon 15 (codons 1862 and 1987) resulted in unstable protein, as determined by Western blotting, and in an AAPC phenotype. Because an unstable protein and a whole-gene deletion might be expected to be functionally equivalent, this difference in disease severity suggests the presence of residual mutant APC protein not detectable by Western blotting in these AAPC patients.

Of our seven *APC*-deletion patients, three had had formal polyp counts at colectomy (25). These counts (800, 1,425, and 1,899) were extremely similar to those typically reported for FAP patients with truncating *APC* mutations between codons 168 and 1250 or codons 1400 and 1580 approximately (26). Perhaps *APC* mutations between codons 168 and 1250 or codons 1400 and 1580 are functionally equivalent to null changes. Germ-line mutations between codons 1250 and 1400, the so-called mutation cluster region (MCR), are associated with more severe colonic polyposis (26, 27), possibly indicating that the mutant protein is more effective at causing tumorigenesis. There are alternative explanations for the phenotypic similarity between patients with *APC* deletions and those with truncating mutations between codons 168 and 1250 or codons 1400 and 1580. For example, the ''second hit'' at *APC* may be rate-limiting for tumorigenesis, and hence the main determinant of disease severity, given the evidence to show that most FAP polyps must acquire a truncating mutation in the MCR for optimal tumorigenesis (28). Germ-line deletion patients and those with truncating mutations outside the MCR must acquire such mutations through a second hit in the MCR, and thus would be expected to have similar disease severity.

The RQM-PCR assay that has been developed for this study proved to be a fast and reproducible method to detect gene dosage at *APC* exon 14. Given its potential for high-throughput analysis, its reliability as well as the small amounts of DNA needed (30 ng per reaction), this assay may be of use in a routine diagnostic setting. In view of the appreciable frequency (11.7%) of *APC* deletions in *APC* mutation-negative classical polyposis patients, this technique may be particularly valuable for this group of patients. Disadvantages of the technique, however, may include the high costs for the detection device and the consumables as well as the laborious assay setup. Although our assay will

detect whole-gene *APC* deletions, it cannot exclude partial deletions 5′ and 3′ of exon 14.

In conclusion, we have developed a RQM-PCR assay to detect *APC* exon 14 deletions. When this technique was applied to a set of apparently *APC* mutation-negative polyposis patients, germ-line deletions were exclusively found in individuals with classical polyposis. Fine-mapping of the region suggested that the majority of these deletions encompass the entire *APC* locus. Given the frequency of 11.7%, screening of *APC* mutation-negative individuals with classical polyposis for germ-line deletions seems warranted.

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