RESEARCH ARTICLE

Promoter-Specific Alterations of *APC* are a Rare Cause for Mutation-Negative Familial Adenomatous Polyposis

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In familial adenomatous polyposis (FAP), 20% of classical and 70% of attenuated/atypical (AFAP) cases remain mutation-negative after routine testing; yet, allelic expression imbalance may suggest an *APC* alteration. Our aim was to determine the proportion of families attributable to genetic or epigenetic changes in the *APC* promoter region. We studied 51 unrelated families/cases (26 with classical FAP and 25 with AFAP) with no point mutations in the exons and exon/intron borders and no rearrangements by multiplex ligation-dependent probe amplification (MLPA, P043-B1). Promoter-specific events of *APC* were addressed by targeted resequencing, MLPA (P043-C1), methylation-specific MLPA, and Sanger sequencing of promoter regions. A novel 132-kb deletion encompassing the *APC* promoter 1B and upstream sequence occurred in a classical FAP family with allele-specific *APC* expression. No promoter-specific point mutations or hypermethylation were present in any family. In conclusion, promoter-specific alterations are a rare cause for mutation-negative FAP (1/51, 2%). The frequency and clinical correlations of promoter 1B deletions are poorly defined. This investigation provides frequencies of 1/26 (4%) for classical FAP, 0/25 (0%) for AFAP, and 1/7 (14%) for families with allele-specific expression of *APC*. Clinically, promoter 1B deletions may associate with classical FAP without extracolonic manifestations. © 2014 Wiley Periodicals, Inc.

INTRODUCTION

Familial adenomatous polyposis (FAP; MIM #175100) is an autosomal dominant disease characterized by hundreds to thousands (classical FAP) or less than one hundred colorectal adenomas (attenuated or atypical FAP, AFAP). Susceptibility to FAP is caused by germline mutations in the adenomatous polyposis coli (*APC*) gene. Some 20% of classical FAP and 70% of AFAP cases remain *APC* mutation-negative (Friedl and Aretz, 2005). This may be due to *APC* alterations that go undetected (Aretz et al., 2007; Spier et al., 2012), nontruncating *APC* alterations whose pathogenic significance is difficult to interpret (Aretz et al., 2004), or predisposing genes other than *APC*, such as *MUTYH* (Sieber et al., 2003) and *AXIN2* (Lammi et al., 2004). Susceptibility to multiple adenomas (more than 10 but less than 100) can also be due to germline mutations in the proofreading domains of *POLE* or *POLD* (polymerase proofreading associated polyposis) (Palles et al., 2013).

The *APC* gene has two promoter regions, 1A and 1B, located 17 and 47 kb upstream of the initiating methionine codon, respectively (Fig. 1). The 1A-specific transcript is considered to be the predominant transcript in normal colon (Horii et al., 1993), although later studies (Rohlin et al., 2011) suggest that promoter 1B may be more important than realized before. We set out to test genetic and epigenetic events in the "major" promoter 1A and the "minor" promoter 1B as explanations for families with *APC*-mutation-negative FAP. We have reported unbalanced allelic expression of *APC* in 33% (7/21) of mutation-negative

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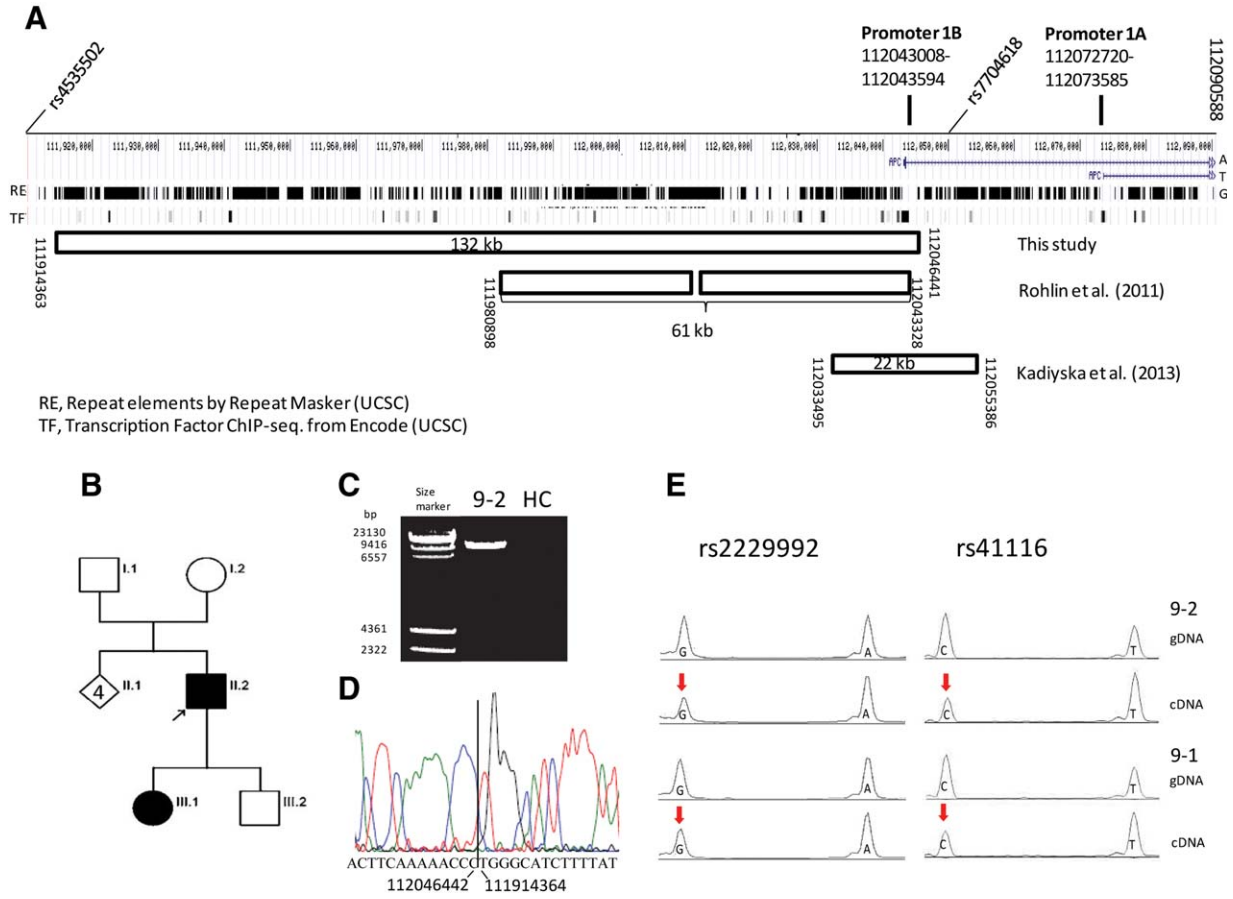

Figure 1. (A) Schematic diagram of *APC* promoter 1B deletions observed to date. The locations are according to GRCh37/Hg19. (B) Pedigree of FAP9. The two patients with polyposis (black symbols), II:2 (9-2) and III:1 (9-1), were investigated. The pedigree has been modified to protect confidentiality. The index patient is indicated by an arrow. (C) Long-range genomic PCR using primers that flank rs4535502 and rs7704618, spanning a region of 140 kb. Genomic deletion resulted in a shortened product (~8 kb) in an affected member (9-2) from FAP9, whereas no visible product was obtained from DNA from a HC. Fragment sizes of a marker ladder are indicated on the left. (D) Sequence tracing that shows the junction formed between the distal and proximal breakpoint of the deletion. The reference sequence used was NC_000005.9 and the mutation was named as c.-176225_c.-44147del132079 relative to the A nucleotide of the initiating ATG. (E) SNuPE analysis of blood genomic DNA (gDNA) and complementary DNA (cDNA) from individuals 9-2 and 9-1 showing expression reduction from the deletion-containing allele. The ratios of allelic peaks (G to A for rs2229992 and C to T for rs41116) in cDNA relative to gDNA were 0.60 and 0.33 for 9-2 and 0.70 and 0.40 for 9-1. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

families with classical FAP (Renkonen et al., 2005), pointing to the likely existence of hidden *APC* alterations. Among 51 families investigated, seven with allele-specific expression (ASE) of *APC* from the previous work were available and of particular interest for the present purposes.

MATERIALS AND METHODS

Patients and Samples

The series consisted of 51 unrelated families/cases, including 26 with classical FAP and 25 with AFAP (Table 1). Based on the method of recruitment, 20 families represented a research-based cohort from Renkonen et al. (2005) lacking *APC* point mutations by sequencing and other techniques and large rearrangements by multiplex ligation-dependent probe amplification (MLPA) P043-B1; the cohort included seven families with unbalanced *APC* expression (indicated in Table 1), eight families showing no ASE (cases 31, 57-1, 78, 88, 92, 97, 104, and 125), and five families uninformative or not included in the allelic expression study. The remaining 31 families represented a prospective clinic-based cohort of index cases with newly diagnosed FAP or AFAP, which remained *APC* mutation-negative after exon-specific sequencing and MLPA P043-B1. Additionally, *MUTYH*-positive cases and occasional cases with mutations in other polyposis-related genes were excluded.

DNA and RNA were extracted from lymphocytes or EBV-transformed lymphoblasts, and

TABLE 1. Clinical and Molecular Characteristics of the Families Investigated

	Case ID[a]	Dominantly inherited[b]	Number of polyps	Age at diagnosis[c]	Extracolonic manifestations[b]	Classification of family[d]	Large rearrangement by MLPA[e]	*APC* methylation by MS-MLPA[f]
RESEARCH BASED	3[g]	Yes	>1000	57	Yes	FAP	No	No
	9-1	Yes	100–1000	20	No	FAP	Promoter 1B deletion	No
	9-2[g]		10–1000	33		(FAP)	Promoter 1B deletion	No
	42[g]	Solitary	100–1000	40	No	FAP	No	No
	63-1	Yes	>1000	50	No	FAP	No	No
	63-2[g]		30	62	No	(FAP)	No	No
	85-1[g]	Yes	>1000	38	Yes	FAP	No	No
	85-2		2000–5000	44	No	(FAP)	No	No
	93[g]	Solitary	>100	58	No	FAP	No	No
	103[g]	Solitary	>100	51	No	FAP	No	No
	31	Yes	>1000	30	Yes	FAP	No	No
	57-1	Yes	100–1000	50	No	FAP	No	No
	57-2		30–40	45		(FAP)	No	No
	78	Solitary	50	55	No	AFAP	No	No
	88	Solitary	100–1000	58	No	FAP	No	No
	92	Solitary	200	51	No	FAP	No	No
	96	Solitary	561	48	No	FAP	No	No
	97	Solitary	>1000	58	No	FAP	No	No
	98	Yes	100–1000	30	No	FAP	No	No
	100	Solitary	30	62	No	AFAP	No	No
	104	Yes?	210	54	Yes	FAP	No	No
	111	Solitary	30–40	36	No	AFAP	No	No
	123	Solitary	2100	37	No	FAP	No	No
	125	Solitary	300	31	No	FAP	No	No
CLINIC BASED	134	Solitary	200–300	55	No	FAP	No	No
	136	Solitary	>100	67	Yes	FAP	No	No
	139	Solitary	100	71	No	FAP	No	No
	145	Recessive	20–50	61	No	AFAP	No	No
	148	Solitary	150–200	50	No	FAP	No	No
	153-1	Yes	50–100	54		FAP	No	No
	153-2		20	37	No	(FAP)	No	No
	158	Solitary	50	49	Yes	AFAP	No	No
	159	Solitary	200	50	No	FAP	No	No
	162	Solitary	>50	52	No	AFAP	No	No
	163	Solitary	10–20	16		AFAP	No	No
	168	Solitary	100	56	Yes	FAP	No	No
	165-1	Yes?	Colon cancer x 2	50		AFAP	No	No
	165-2		20–30	33	Yes	(AFAP)	No	No
	1001	Yes	10	48		AFAP	No	No
	1003	Solitary	20–30	70		AFAP	No	No
	1005	Yes	10–20	68	Yes	AFAP	No	No
	1006	Solitary	20	60	No	AFAP	No	No
	1007	Solitary	20	30	No	AFAP	No	No
	1010	Yes	5–10	68		AFAP	No	No
	1011		60–100	31		FAP	No	No
	1013	Solitary	>100	48		FAP	No	No
	1015	Solitary	10	47	Yes	AFAP	No	No
	1017	Solitary?	10–20	57		AFAP	No	No
	1018	Solitary	20–30	74		AFAP	No	No
	1019	Solitary	2–3	30	Yes	AFAP	No	No
	1020	Solitary	3	35	Yes	AFAP	No	No
	1021	Solitary	30	72		AFAP	No	No
	1022	Yes	3	65	Yes	AFAP	No	No
	1023	Solitary	40	33		AFAP	No	No

TABLE 1. (Continued)

Case ID[a]	Dominantly inherited[b]	Number of polyps	Age at diagnosis[c]	Extracolonic manifestations[b]	Classification of family[d]	Large rearrangement by MLPA[e]	*APC* methylation by MS-MLPA[f]
1024	Solitary	20	72		AFAP	No	No
1025	Solitary	20–30	67		AFAP	No	No
1026	Solitary	10–20	51		AFAP	No	No

[a]Identification number of family, followed by identification number of individual if several members were investigated per family.
[b]Indicated for cases with available data.
[c]Polyposis or colorectal carcinoma, whichever comes first.
[d]Based on the highest number of adenomas in the family (may include information of members not shown).
[e]P043-C1.
[f]ME001-C1.
[g]ASE cases from Renkonen et al. (2005).

normal and tumor DNA from formalin-fixed paraffin-embedded tissues as described in Renkonen et al. (2005). This study was approved by the institutional review board of the Helsinki University Central Hospital (Helsinki, Finland).

Next-Generation Sequencing

A 0.9 Mb region around *APC* (111,772,101–112,672,101/Hg19) was targeted by the SureSelect Target Enrichment system (Agilent Technologies) and used for paired-end resequencing on the SOLiD platform (Applied Biosystems). Disease-associated and healthy haplotypes were constructed based on all SNPs identified in the region. Specifically, the Life Technologies LifeScope software (v2.1) with default settings were used for aligning the raw reads against the human reference genome (hg18 assembly originally) and for variant calling annotation based on the dbSNP build 130. There were 29M and 36M mapped reads for the human samples, and the average read coverage was around 3750. Less than 0.07% of the target regions were not covered by any reads and 99.8% of the targets were covered by more than 20 reads in all samples depicting high resolution of the analysis. Next-generation sequencing and bioinformatic analysis for SNPs were performed at Turku Centre for Biotechnology, University of Turku and Åbo Akademi University, Turku, Finland.

Multiplex Ligation-Dependent Probe Amplification

SALSA MLPA probemix P043-C1 for *APC* was used according to the manufacturer's instructions (MRC-Holland, the Netherlands; http://www.mlpa.com) on 250 ng of template DNA. Amplification products were visualized by fragment analysis carried out on the Applied Biosystems ABI 3730 Automatic DNA Sequencer and analyzed with Applied Biosystems GeneMapper 4.0 genotyping software and/or analysis tool (Coffalyser) provided by the manufacturer.

Single Nucleotide Primer Extension (SNuPE)

The basic principle of SNuPE is described in Renkonen et al. (2005). Four coding polymorphisms within *APC*—rs2229992 (exon 11), rs351771 (exon 13), rs459552 (exon 15), and 3'UTR (rs41116)—were utilized to design gDNA and cDNA-specific PCR reactions. PCR products served as templates for primer extensions with three dNTPs and one ddNTP, resulting in products of different sizes for the two alleles at a given SNP position. Details of the SNuPE reactions, including the sequences for the amplification and extension primers, are given in Supporting Information Table 1 (if the same primers were used for gDNA and cDNA, RNA was DNase-treated prior to conversion into cDNA).

Quantitative Reverse Transcription PCR (qRT-PCR)

To assess total *APC* expression, quantitative RT-PCR was carried out with Taqman Gene Expression Assay (Applied Biosystems) for probe APC4 (Hs01568270_m1), which detects transcripts generated from promoters 1A and 1B (Rohlin et al., 2011). Human *GUSB* (Hs00939627_m1) and *TBP* (Hs00427621_m1) were used as references. The assays were run in triplicate on 7500 Fast Real-Time PCR system (Applied Biosystems). Expression levels of *APC* were quantified by normalization to the reference genes and calculated by the $2^{-\Delta\Delta CT}$ method.

Long Range Genomic PCR

To isolate the segment deleted in FAP9, long-range genomic PCR was conducted with primers

5′-GCAAATGCAATTGGTGTGTT-3′ (forward) and 5′-CAGTTATCATACACTACTATGCC-3′ (reverse), flanking rs4535502 and rs7704618, respectively. The PCR product was run on a 1% agarose gel, cut out, purified, and used as a template for primer walking.

Sequencing of *APC* Promoter 1A and 1B for Point Mutations

An 856-bp region around promoter 1A (GenBank accession U02509) was sequenced in two overlapping fragments using primers Fw1: 5′-CAGTGACACCCTGGCGGGCTG-3′ + Rv1: 5′-GCTAGCATAGCTTTTCTGGTAAC-3′ and Fw2: 5′-AAGTCACTGAGTTGTCAGAGTGA TG-3′ + Rv2: 5′-GGATTTTGTCCTTCAACCT CA-3′. A 793-bp region around promoter 1B (GenBank accession D13981) was sequenced in two overlapping fragments with primers Fw3: 5′-GCCAGGAAAGGTGGAGGAC-3′+ Rv3: 5′-GTGAGAGGTGTTGCTGGCTT-3′ and Fw4: 5′-CCCACAGCCCGGAGACTAGAGCCTG-3′ + Rv4: 5′-AGGCCAGTAAGTGCTGCAACTGAGACT-3′.

Methylation Analysis

The methylation status of *APC* promoter 1A was investigated by methylation-specific MLPA (MS-MLPA) using the SALSA MS-MLPA ME001-C1 Tumor suppressor-1 kit (MRC-Holland, the Netherlands; http://www.mlpa.com) following the manufacturer's instructions. We used 100–150 ng of template DNA, and amplification products were visualized by fragment analysis as described above for MLPA. Methylation dosage ratios were calculated as specified (Gylling et al., 2008), and a dosage ratio of 0.15 or higher (corresponding to 15% of methylated DNA) was considered to indicate promoter methylation. The methylation status of promoter 1B was studied by bisulfite sequencing as described (Romero-Giménez et al., 2008).

RESULTS AND DISCUSSION

Identification of a Novel Deletion Affecting Promoter 1B

For a comprehensive evaluation of *APC* and flanking regions for genetic alterations, three families with ASE were selected for targeted resequencing of a 0.9 Mb region around *APC*, as described in Materials and Methods. An unusually long stretch of homozygosity/hemizygosity for SNPs (~140 kb flanked by heterozygous markers rs4535502 and rs7704618, Fig. 1A) was shared by two affected members from an unbalanced expression family (FAP9; Fig. 1B). Long-range genomic PCR utilizing the flanking markers as anchors revealed a ~8 kb fragment in an affected family member, whereas no product was generated from a healthy control (HC; Fig. 1C). A junction fragment defining the exact deletion breakpoints was subsequently identified by primer walking (Fig. 1D). The deletion spanned 132 kb including the entire promoter 1B and upstream sequence. While our study was in progress, Rohlin et al. (2011) described a 61 kb deletion in a FAP family from Sweden and Kadiyska et al. (2013) a 22 kb deletion in a FAP family from Bulgaria. The locations of all three deletions involving promoter 1B reported to date are depicted in Figure 1A. Each deletion is novel and removes part (Rohlin et al., 2011) or the entire promoter 1B (Kadiyska et al., 2013; present study). The major transcription factor sites that are lost in the deletion herein are listed in Supporting Information Table 2. The deletions could be facilitated by the abundance of repetitive sequences in the flanking regions, although without apparent homology between sequences flanking the actual breakpoints (Kadiyska et al., 2013; present study).

The deletion in FAP9 turned out to be detectable by MLPA (P043-C1), giving rise to a ~50% peak area reduction for two probe pairs corresponding to promoter 1B (Fig. 2). We subsequently used the MLPA P043-C1 test to screen the remaining mutation-negative FAP and AFAP families for possible rearrangements. No additional cases with promoter 1B deletion were found. No isolated rearrangements of promoter 1A were present in our series, either. While deletions involving promoter 1A are not uncommon *per se*, these typically affect the *APC* coding region as well (Renkonen et al., 2005; Rohlin et al., 2011), and cases with large rearrangements in the coding region, including deletions that extended to promoter 1A, were excluded at the outset. Charames et al. (2008) described a single family with possible promoter 1A-specific deletion associated with complete silencing of the deletion-containing allele. The deletion was diagnosed by MLPA P043-B1, which reliably excludes copy number alterations in the *APC* coding region; however, owing to the lack of promoter 1B-specific probes, it remains unsettled whether or not the deletion also affected promoter 1B.

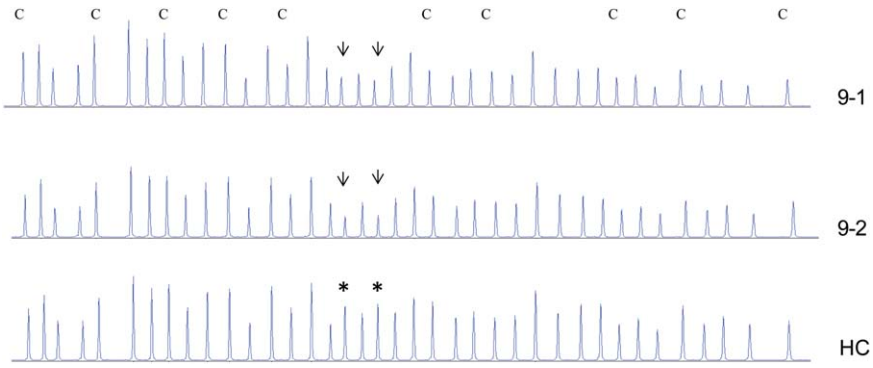

Figure 2. Outcomes of the P043-C1 MLPA assay in the polyposis patients 9-1 and 9-2 as well as a HC individual. As indicated by arrows, both promoter 1B-specific peaks included in the assay were reduced to ~50% in the polyposis patients compared to the same peaks (asterisks) in the HC. Peaks of the control probes (from genomic regions unrelated to *APC*) are marked with C. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Allele-Specific Expression of *APC*

SNP-based haplotypes representing the affected and unaffected alleles were constructed and utilized to design primer extension analyses for allelic expression in FAP9 (Fig. 1E). All four coding SNPs tested from different regions of *APC* (rs2229992 from exon 11, rs351771 from exon 13, rs459552 from exon 15, and rs41116 from 3′UTR) showed a consistent relative expression reduction from the deletion-containing allele of the two affected family members, with the average allelic ratios in cDNA relative to genomic DNA varying between 0.4 (60% reduction) and 0.6 (40% reduction) depending on the SNP (a ratio around 1 would indicate balanced expression). The degree of ASE in FAP9 is somewhat milder than that reported for the Bulgarian family (70% reduction for one of the two alleles; Kadiyska et al., 2013) and clearly lower compared with the Swedish family (91% reduction; Kanter-Smoler et al., 2008; Rohlin et al., 2011). Lymphoblastoid RNA from individual 9-1 was available for qRT-PCR analysis of total *APC* expression relative to HC s, and the results were compatible with SNuPE (30–50% reduction in the average *APC* expression depending on the assay setup). It is unknown at present if the extent of the deletion or the allelic background might influence the severity of expression consequences associated with promoter 1B deletions. Based on the alleles showing reduced expression, the deletions in the Swedish, Bulgarian, and Finnish FAP families were not confined to a particular ancestral haplotype.

Loss of Heterozygosity in Tumor Tissue

DNA samples from two colorectal adenomas, one from each investigated member of FAP9, were available for loss of heterozygosity (LOH) analysis by the rs2229992-based SNuPE. One adenoma did not show LOH (Renkonen et al., 2005); other possible "second hits" (such as somatic point mutations or hypermethylation) were not tested. The other adenoma showed partial LOH of the wild-type allele (Supporting Information Fig. 1). Unlike the Swedish family in which no second hits were found (Rohlin et al., 2011) our findings support the two-hit mechanism of inactivation in association with promoter 1B deletion.

Analysis of Promoters 1A and 1B for Point Mutations

To detect possible point mutations that might affect *APC* expression in an allele-specific fashion, nearly 1 kb regions around promoters 1A and 1B were sequenced in the seven families with unbalanced expression. No alterations suspected to be pathogenic were found. The rarity of point mutations in our series is in agreement with findings obtained in *APC*-mutation negative polyposis from other populations (Heinimann et al., 2001).

Promoter Methylation

In colorectal carcinomas, methylation of promoter 1A is present in 20 – 45% of tumors (Esteller et al., 2000; Joensuu et al., 2008; Segditsas et al., 2008) whereas promoter 1B is not prone to aberrant methylation (Esteller et al., 2000). While preliminary evidence (Hitchins et al., 2006; Romero-Giménez et al., 2008) does not support a role for *APC* promoter hypermethylation in mutation-negative FAP, it was

relevant to test promoter hypermethylation in our series, as cases with unbalanced *APC* expression have not previously been investigated in that regard. All 51 families/cases were screened for constitutional hypermethylation of promoter 1A by MS-MLPA. No hypermethylation at the *APC* promoter 1A or the promoters of any other 23 tumor suppressor genes included in the MS-MLPA ME001-C1 test was present in blood DNA from any case irrespective of ASE. Moreover, bisulfite sequencing revealed no aberrant methylation at promoter 1B in the seven ASE families tested. Tumor studies have found that the effect of *APC* promoter 1A hypermethylation on expression may be too subtle to qualify for an inactivating "hit" (Segditsas et al., 2008) and the lack of constitutional epimutations that would cause susceptibility to FAP is compatible with this notion.

CONCLUDING REMARKS

In summary, a comprehensive screen of 51 mutation-negative FAP families for promoter-specific events (large rearrangements, point mutations, and hypermethylation) of *APC* resulted in the identification of a pathogenic alteration in one family. We describe a novel 132 kb deletion involving promoter 1B as the cause for ASE in two members from the affected family. The phenotype of classical FAP with no extracolonic manifestations appears common to all three families with promoter 1B deletions reported to date (Table 1; Rohlin et al., 2011; Kadiyska et al., 2013). We conclude that isolated promoter-specific events are not a frequent cause of *APC* mutation-negative FAP overall, but promoter 1B deletions are a worthwhile possibility especially if unbalanced expression of *APC* alleles is evident.

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