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APC promoter 1B deletion in seven American families with familial adenomatous polyposis

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Abstract

Familial adenomatous polyposis (FAP) is a colorectal cancer predisposition syndrome caused by mutations in the adenomatous polyposis coli (*APC*) gene. Clinical genetic testing fails to identify disease causing mutations in up to 20% of clinically apparent FAP cases. Following the inclusion of multiplex ligation-dependent probe amplification (MLPA) probes specific for *APC* promoter 1B, seven probands were identified with a deletion of promoter 1B. Using haplotype analysis spanning the *APC* locus, the seven families appear to be identical by descent from a common founder. The clinical phenotype of 19 mutation carriers is classical FAP with colectomy at an average age of 24. The majority of cases had a large number of duodenal and gastric polyps. Measurements of allele-specific expression of *APC* mRNA using TaqMan assay confirmed that relative expression was reduced from 98% to 42% in the allele containing the promoter 1B deletion, depending on tissue type. This study confirms the importance of *APC* promoter deletions as a cause of FAP and identifies a founder mutation in FAP patients from the United States.

Keywords

familial adenomatous polyposis; *APC* promoter 1B; colon cancer; allelic imbalance; founder mutation; *APC*

INTRODUCTION

Familial Adenomatous Polyposis (FAP; [MIM 175100]) is an autosomal dominant colorectal cancer predisposition syndrome characterized by hundreds to thousands of precancerous colonic adenomatous polyps with an average onset between the late teens and the early thirties. Patients are also at increased risk for gastric, duodenal, pancreatic, thyroid, and CNS tumors (1, 2). The severity of polyposis and presence of extra-colonic features

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depend, in part, on the position of the *APC* mutation (2). To date, over 1000 unique likely pathogenic *APC* mutations have been reported in FAP and attenuated FAP (AFAP) patients (Leiden Open Variation Database; http://chromium.liacs.nl/LOVD2/colon_cancer/home.php?select_db=APC). In approximately 10% of FAP cases and potentially more than 50% of AFAP cases, the mutation eludes identification (3). Mutations in other causative genes like *MUTYH* and in *APC* noncoding regions, such as intronic mutations which disrupt correct exonic splicing, combined with older testing technologies account for some of the undetected mutations. Specific to this issue is the introduction of multiplex ligation-dependent probe amplification (MLPA) for detection of large deletions in exons, and most recently, promoter 1B of *APC* (4). With the introduction of MLPA, large but submicroscopic genomic *APC* deletions were more readily detected and are now recognized to constitute around 12% of FAP cases (3). To our knowledge, three groups have identified and characterized distinct large deletions encompassing the *APC* promoter 1B (5–7). We report of seven FAP families residing in the United States with an *APC* promoter 1B deletion who share identity by descent at the *APC* locus, suggesting they come from a common founder.

METHODS

Research Subjects

This study was approved by University of Utah's Institutional Review Board. Research participants were enrolled into the Hereditary Gastrointestinal Cancer Registry. The registry includes 244 families with FAP or AFAP (122 mutation identified, 24 no mutation identified, 98 *APC* not tested). Medical records were reviewed for colonic polyp burden, history of colectomy, gastric polyposis, duodenal polyposis, and extracolonic features associated with FAP. The severity of duodenal polyposis was scored and staged by modified Spigelman classification (8). When descriptors were ambiguous ("several", "numerous", "multiple", "few") a modest estimation of 1 point was scored for number of polyps.

Haplotype analysis

Genomic DNA was genotyped by PCR amplification at four short tandem repeat (STR) loci (D5S2501, D5S2027, D5S346, and D5S421) which span the *APC* locus using previously published reaction conditions (9). Allele frequencies used to calculate the probability of inheriting the specific haplotype were based on those reported by the Centre d'Etude du Polymorphisme Humain (CEPH) genotype database browser (<http://www.cephb.fr/en/cephdb/browser.php>), derived from a minimum of 27 unrelated Caucasian individuals from eight kindreds (10).

Genetic testing

MLPA deletion/duplication analysis was performed on genomic DNA from peripheral blood samples using SALSA MLPA *APC* probe mix and reagent kit (MRC-Holland #P043-*APC*, LOT#1212, version C1; MRC-Holland #EK1-FAM) according to the manufacturer's protocol. Fragment separation was performed using an Applied Biosystems-3730 DNA Analyzer. Coffalyser.Net software provided by MRC-Holland was used for data analysis.

COLARIS-AP[®] clinical genetic testing was performed on the proband in 2 of our 7 kindreds under standard of care (Myriad Genetics).

HumanOmniExpressExome BeadChip was run on an iSCAN instrument and SNP alleles were called using GenomeStudio software (Illumina).

Deletion specific PCR for the Bulgarian 22 kb promoter 1B mutation was done using conditions provided by the authors (5). Primers used were:

F1:GCTGATTATTGCATTCAGCC (centromeric to deletion: g.112,033,309-112,033,328),
R1:ATAGGAGATGGGTAGGACGG (telomeric to deletion: g.112,056,201-112,056,220),
R2:GTTCCCTGCCCAATAACTGC (internal to deletion: g.112,033,971-112,033,990) with an annealing temperature of 58°C and 1.5 mM MgCl₂.

mRNA and cDNA generation

RNA expression was measured from endoscopic biopsies immediately placed in *RNALater*[™]. Total RNA was prepared using Qiagen RNeasy RNA purification kits (Qiagen #74104). Cell line mRNA was purified using an RNAeasy Mini Kit (Qiagen #74106) following the provided protocol including the on-column RNase-free DNase treatment. Blood samples were collected in PAXgene Blood RNA Tubes (BD #762165) and stored at -80°C. The RNA was purified using a PAXgene Blood miRNA Kit (Qiagen #763134) followed by RNA Cleanup protocol using an RNAeasy Mini Kit. cDNA was synthesized using a SuperScript VILO cDNA Synthesis Kit (Invitrogen #11754).

mRNA analysis

Expression from the promoter 1B deletion allele was determined by measuring cDNA for relative levels of the APC SNP rs459552 (T* > A) using a TaqMan SNP Genotyping Assay with the TaqMan Genotyping Master Mix (Life Technologies # C_3162935_20 and # 4371357) following manufacturer's recommended conditions. Genomic DNA from each individual was also run in order to normalize the difference in signal intensity between T- and A-alleles. Assays were performed on a Bio-Rad CFX96 Real-Time PCR System in duplicate allowing all samples to be run in a single 96-well plate to avoid variability. The 2^{-CT} method was used to calculate the T*/A expression ratio from cDNA samples and normalizing with the overall average C_T of genomic DNA.

RESULTS

Identification of related FAP families based on APC haplotype

University of Utah hosts a hereditary gastrointestinal cancer registry that includes over 600 individuals from 244 kindreds with FAP or AFAP. The APC gene has been sequenced in individuals representing 146 of these kindreds (122 with a deleterious mutation, 24 with no mutation identified). The 98 remaining kindreds have only a clinical diagnosis; genetic testing is not reported. The number without a mutation detected is not surprising because many probands are referred to our research registry when a genetic diagnosis cannot be made.

When new and often novel mutations are identified in *APC*, we have the opportunity to determine if other kindreds in our registry descend from a common founder harboring the mutation using a relatively simple approach. *APC*-specific genotypes using four highly heterozygous STR markers (9) have been generated for 24 FAP families who previously tested negative for mutation in *APC* and 35 FAP families with available DNA who had never been tested for mutations in *APC*, creating an ancestral fingerprint across the *APC* genomic region. We report here a specific application of this concept whereby an *APC* promoter 1B deletion was recently identified through clinical testing in a registry participant (K44-9; Table 1). Using the STR genotypes from our registry, 6 additional kindreds were found to share the same four K44 STR markers at D5S2501, D5S2027, D5S346, and D5S421 with alleles 3, 2, 4 and 4 and population frequency 0.365, 0.308, 0.021, and 0.167 respectively (Table 1). The product of the haplotype frequencies of the four STR marker alleles is 0.00039 or approximately 1:2536, suggesting that all 7 kindreds descend from a common ancestor.

Deletion analysis

MLPA was run on all 58 registry kindreds with STR data plus K44. As predicted, MLPA testing showed a deletion of the two probes that map to promoter 1B of *APC* in the probands of all 7 kindreds: K8, K43, K44, K256, K509, K685, and K691 (Table 1). These seven represent four of 24 families that previously tested negative for *APC* mutations and three of 35 families that had never been tested for *APC* mutations. Twelve additional affected family members showed the deletion and one unaffected member did not show the deletion (Table 1 and Figure 1). SNP genotyping using HumanOmniExpressExome defines the maximum boundaries by heterozygous SNPs at rs12719151 and rs10075281 as g.chr5: [112,026,480]+?_-[112,060,242] based on hg19/37. No other promoter specific deletions were identified; however one kindred showed a deletion of the full *APC* gene including the promoters.

The MLPA analysis reveals that this promoter 1B mutation is distinct from that reported in families from Sweden (6); we would expect the Swedish mutation to show a deletion of only the 5' most (260nt) probe by MLPA. Our reported deletion at approximately 34 kb is smaller than the 132 kb deletion from Finland (7). It overlaps however with the Bulgarian deletion of 22 kb (5). We thus tested our promoter 1B deletion samples using a deletion-specific PCR for this mutation (5). Primers flanking the deletion (F1+R1) produce a deletion-specific product of 1019 bp, which is not seen in our samples (data not shown). Nested primers (F1+R2) produce a 682 bp product from the wild-type *APC* allele, which is the only band observed from our samples. These results indicate that our families do not have the Bulgarian deletion.

Gastrointestinal phenotype

To verify the clinical status, endoscopic and genetic data were collected on additional family members. Mutation carriers had a colonic phenotype consistent with classic FAP (Table 1) and similar to previous reports (5–7). Most individuals had undergone colectomy (n=17 of 19 with records) at an average age of 24.2 years. Esophagogastroduodenoscopy records (n=16 of 19) showed that gastric and duodenal polyps were common. Two patients with

Spigelman stage IV required duodenectomy at ages 50 and 30. Although extracolonic data was limited, a desmoid was reported in one individual from K43, a hepatoblastoma was reported in a 9 month old child from K44, and epidermoid cysts were reported in K44. Although osteomas are reported in the Swedish family (6), we did not find osteomas in 3 available reports. There were also 9 negative and one positive report of thyroid nodules, and 7 reports of normal teeth.

Allelic imbalance

The *APC* gene has two promoters, 1A and 1B, which generate different mRNA products and appear to undergo tissue-specific regulation (6, 11). We were interested in determining the effect of the 1B promoter deletion on *APC* transcript levels relative to the wild-type allele. Samples were analyzed from individuals harboring the promoter 1B mutation and heterozygous for a common SNP in *APC*, rs459552 (T* > A). mRNA from blood, EBV transformed lymphoblast cell lines, and endoscopic biopsies from normal duodenal tissue were quantitated using a TaqMan assay (Figure 2). When normalized to genomic DNA and compared with mRNA from the wild-type *APC* gene (A-allele), the total mRNA from the mutant *APC* gene (T-allele) is down an average of 42% in cell lines (n=4), 88% in normal duodenal tissue (n=4), and 98% in blood (n=3). These results are similar to previous reports with 91–70% reduction in blood (5, 6) and 40–60% reduction in lymphocytes or cell lines (7). It is presumed that the residual mRNA expression from the T-allele arises from promoter 1A. Expression from promoter 1B is estimated to be considerably higher than 1A: 15–25 fold in gastric mucosae (11), and 100-fold in blood (6). This fits well with our observations, which show near-complete loss of the mutant allele in blood. By comparison, *APC* mRNA from Lynch syndrome or control patients did not show a major reduction.

DISCUSSION

We report identification of an *APC* promoter 1B deletion in seven independently ascertained families in the United States that, in all likelihood, descend from a common founder. Connecting the genealogy of the 7 families and defining the precise deletion boundaries are pieces of the puzzle that will need to be solved. These individuals present with a classic FAP phenotype featuring severe colonic and upper gastrointestinal polyposis. The seven families represent approximately 3% of the families in our large hereditary gastrointestinal cancer registry, and 16% of those that previously tested negative for an *APC* mutation. The prevalence of this mutation and the severity of polyposis is potentially over estimated because referral registries such as ours are weighted with the more extreme cases.

We also present an approach of using STR genetic markers as a reliable technique to effectively cross-reference and identify samples that are genetically identical by descent at the locus of interest. This provides a cost-effective methodology for identifying families that may share a newly-described pathogenic mutation in any number of genes.

APC promoter 1A is down-regulated through hypermethylation in healthy gastric mucosa and gastric cancers (12). Unlike the colonic phenotype, duodenal and gastric polyps are not universal in FAP patients, with duodenal adenomas reported in 50–90% and fundic gland polyposis in 40–50% of patients (2). We speculate that the loss of expression from the *APC*

promoter 1B deletion allele combined with low levels of promoter 1A expression may modify the presence of gastric and duodenal polyps. Duodenal and gastric polyps were observed in most, but not all, of our promoter 1B deletion carriers. Further study of *APC* promoter 1A versus 1B expression in these as well as other FAP patients relative to their upper gastrointestinal phenotype would provide important insights into regulation of *APC* and its role in gastric and duodenal carcinogenesis.

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Abbreviations

FAP	Familial adenomatous polyposis
APC	adenomatous polyposis coli
STRs	short tandem repeats
MLPA	multiplex ligation-dependent probe amplification

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Figure 1. Identification of *APC* promoter 1B deletion by MLPA

Representative MLPA results for three individuals from two of the families: K509 (affected and unaffected) and K44 (affected). The affected individuals show deletion of the two promoter 1B probes, APC-1 260nt and APC-1 274nt, but not the promoter 1A probes (APC-2), nor any of the coding exons (APC-4 through -18). *APC* exon numbering is based on Genbank reference sequence NG_008481.4 wherein the ATG translation start site is located in exon 4. Probes cover *MUYTH* (first 3 data points in each panel), *APC* (middle 25 data points shown in orange in each panel), and genomic reference sequences (last 10 data points in each panel).

Figure 2. Reduced mRNA expression from allele with *APC* promoter 1B deletion in different tissues

TaqMan SNP Genotyping Assay for rs459552 was used to measure allele specific mRNA expression. Average expression of the T*-allele, linked to the *APC* promoter 1B deletion, relative to the A-allele, linked to the wild-type *APC* promoter, from three tissue mRNA sources is reported. A sample of genomic DNA (gDNA) from each individual was also run in order to normalize the difference in signal intensity between T*- and A-alleles as detailed in Methods. Error bars represent the range of values. The average gDNA T*/A-ratio normalized to itself and the range of ratios for 13 gDNAs run are shown in the first data point. Control and Lynch syndrome cDNAs are represented as light grey bars. *APC* promoter 1B deletion cDNAs are represented as dark grey bars. Numbers following the kindred refer to individuals in Table 1. Note that blood mRNA for K44-10 was also tested, but could not be plotted because T*-allele expression was below assay detection levels.

Table 1

Clinical Phenotype of APC Promoter 1B Mutation Carriers

Kindred	Individual	Gender	Lower Phenotype		Upper Phenotypes			APC STR Haplotypes			
			Number Colon Polyps (age)	Age @ Colectomy	Number Gastric Polyps (age)	Number Duodenal Polyps (age)	Spigelman Stage	D5S2027 (2)	D5S2501 (3)	D5S346 (4)	D5S421 (4)
8	1	F	ND	27	hundreds (41)	3 (41)	I	2, 2	2, 3	4, 11	2, 4
	2	F	ND	24	2 (50)	1 (50)*	IV	2, 2	3, 3	4, 5	3, 4
8	3	M	hundreds (16)	16	150 (18)	10 (17)	III	2, 4	3, 3	4, 5	3, 4
43	4	F	ND	18	30 (39)	20 (39)	II	1, 2	3, 5	4, 6	4, 4
43	5	F	ND	ND	multiple (46)	several (46)	II	1, 2	3, 4	4, 6	4, 4
44	6	F	hundreds (39)	40	hundreds (39)	multiple (39)	I	ND	ND	ND	ND
44	7	F	ND	ND	ND	ND	ND	2, 4	2, 3	4, 12	3, 4
44	8	F	hundreds (47)	48	normal (46)	normal (46)	0	ND	ND	ND	ND
44	9	M	>300 (26)	NA	150–200 (28)	7 (28)	I	2, 4	3, 4	4, 9	2, 4
44	10	M	thousands (22)	23	multiple (22)	normal (22)	0	2, 4	3, 4	4, 9	2, 4
256	11	M	ND	37	>500 (54)	49 (54)	II	2, 5	3, 5	4, 6	4, 5
509	12	F	ND	21	numerous (60)	numerous (60)	II	2, 5	3, 3	4, 10	2, 4
509	13	M	NA	NA	NA	NA	NA	3, 6	2, 3	10, 12	2, 3
509	14	F	ND	28	ND	ND	ND	2, 3	3, 3	4, 12	2, 4
685	15	F	ND	27	35 (56)	17 (56)	III	1, 2	3, 3	4, 5	3, 4
685	16	M	40–50 (14)	15	multiple (14)	nodularity (14)	I	2, 3	3, 3	4, 9	3, 4
685	17	M	multiple (13)	14	multiple (13)	a few (13)	I	2, 3	3, 3	4, 9	3, 4
685	18	F	ND	15	200+ (40)	30 (40)	III	2, 2	3, 4	4, 6	4, 4
685	19	F	ND	33	ND	ND	ND	1, 2	3, 3	4, 5	3, 4
685	20	F	ND	16	75 (29)	6 (30)	IV	ND	ND	ND	ND
691	21	F	ND	10	1 (50)	multiple (50)	II	1, 2	3, 3	4, 10	4, 4

ND = no data; NA = not applicable; asterisk (*) denotes individual who had multiple large duodenal polyps over time who subsequently underwent surgery at age 52 for a 15mm villous adenoma and at age 62 for a 20mm tubular adenoma with areas of neuroendocrine neoplastic cells. APC haplotype segregating with promoter 1B deletion noted in parenthesis (2), (3), (4), (4)