Multiple Rare Nonsynonymous Variants in the *Adenomatous Polyposis Coli* Gene Predispose to Colorectal Adenomas

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Abstract

It has been proposed that multiple rare variants in numerous genes collectively account for a substantial proportion of multifactorial inherited predisposition to a variety of diseases, including colorectal adenomas (CRA). We have studied this hypothesis by sequencing the *adenomatous polyposis coli* (*APC*) gene in 691 unrelated North American patients with CRAs and 969 matched healthy controls. Rare inherited nonsynonymous variants of *APC* were significantly overrepresented in patients who did not carry conventional pathogenic mutations in the *APC* or *MutY homologue* genes [non–familial adenomatous polyposis (FAP) non–*MUTYH*-associated polyposis (MAP) patients; 81 of 480, 16.9%] compared with patients with FAP or MAP (20 of 211, 9.5%, $P = 0.0113$), and this overrepresentation was highest in those non-FAP non-MAP patients with 11 to 99 CRAs (30 of 161, 18.6%, $P = 0.0103$). Furthermore, significantly more non-FAP non-MAP patients carried rare nonsynonymous variants in the functionally important β-catenin down-regulating domain compared with healthy controls (32 of 480 versus 37 of 969, $P = 0.0166$). *In silico* analyses predicted that ~46% of the 61 different variants identified were likely to affect function, and upon testing, 7 of 16 nonsynonymous variants were shown to alter β-catenin–regulated transcription *in vitro*. These data suggest that multiple rare nonsynonymous variants in *APC* play a significant role in predisposing to CRAs. [Cancer Res 2008;68(2):358–63]

Introduction

In support of the "rare variant hypothesis" of multifactorial inherited predisposition to common diseases (1), it has recently been shown that rare nonsynonymous variants in the genes encoding apolipoprotein A1, the ATP-binding cassette transporter A1 and lecithin cholesterol acyltransferase, are overrepresented in individuals with low plasma levels of high-density lipoprotein cholesterol, a major risk factor for coronary atherosclerosis (2). It is likely that rare nonsynonymous variants in numerous genes also predispose to colorectal tumors because epidemiologic studies predict that inherited factors play a role in 15% to 30% of colorectal cancers (CRC), but only a minority of these can be accounted for by established CRC predisposition alleles (1, 3). Germline truncating mutations in the *adenomatous polyposis coli* (*APC*) gene cause familial adenomatous polyposis (FAP; MIM 175100), an autosomal dominant disorder characterized by hundreds or thousands of colorectal adenomas (CRA), some of which progress to cancer (4) and inherited mutations in the human MutY homologue (*MUTYH*) gene cause *MUTYH*-associated polyposis (MAP; MIM 608456), an autosomal recessive disorder with a multiple CRA and CRC phenotype (5, 6). Whether rare inherited nonsynonymous variants in *APC* might act as low penetrance alleles remains highly speculative; however, the variant I1307K has been shown to create a hypermutable tract that predisposes to somatic mutations (7) and E1317Q has been shown to be overrepresented in the germline of patients with multiple CRAs (8) and has also been identified as a somatic change in sporadic CRC (9).

To address the potential role of rare nonsynonymous variants of *APC* in inherited predisposition to CRAs, we sequenced *APC* in 691 unrelated North American patients with CRAs and 969 matched healthy controls. We found 61 different rare inherited nonsynonymous variants and showed that these variants were significantly overrepresented in patients who did not carry conventional pathogenic mutations in *APC* or *MUTYH*. Functional and *in silico* analyses supported the genetic data. We conclude that many such variants do indeed predispose to CRAs providing a potential mechanism to identify individuals at increased risk of CRC.

Materials and Methods

Patients and control samples. We undertook comprehensive mutation analysis of the *APC* and *MUTYH* genes in 691 unrelated North American patients that were referred by their physicians for genetic testing because of a clinical diagnosis of either FAP or "multiple" colorectal polyps. All results were made completely anonymous in accordance with institutional approved guidelines. We also sought rare nonsynonymous variants in residual DNA samples from 969 unrelated North American healthy controls that were made available after routine carrier screening for cystic fibrosis. Samples were made completely anonymous in accordance with institutional policies governing specimen use. No samples carried any of the 97 mutations that were tested for in the *CFTR* gene. The unrelated North American healthy controls were matched to the unrelated North American non-FAP non-MAP patients for age (mean, 41.6 years for controls and 47.8 years for patients), sex (~50% males/50% females in both groups), and self-reported ethnic backgrounds (Supplementary Table S1).

Mutation analyses. Peripheral blood DNA samples from all patients were sequenced for the entire open reading frame (ORF) and splice sites of *APC* and exons 7 and 13 of *MUTYH* (which harbor the two common *MUTYH* mutations; ref. 6) and screened for deletions at the *APC* locus by MLPA and by Southern blot analysis. Samples with a single *MUTYH*

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Table 1. Rare inherited nonsynonymous variants spanning the *APC* ORF in 691 North American patients with CRAs (classified according to FAP/MAP status and number of adenomas) and in the β-catenin down-regulating domain in 969 North American healthy controls

Category	Nonsynonymous variants	Total (frequency)
FAP/MAP patients		
(*APC* ORF)		
FAP patients	R106H, A199T+L639S, R414C+G2502S, S537C, L1129S, I1307K (2), E1317Q (2), R1676G+P2467T, H2149P, A2274V, G2502S (3), R2505Q, S2621C	17 of 178 (9.6%)
MAP patients*	E1317Q, G2502S (2)	3 of 33 (9.1%)
Total		20 of 211 (9.5%) †
Non-FAP non-MAP patients		
(*APC* ORF)		
≤10 CRAs	P981R, I1307K (2), E1317Q (2), T1445A, G2502S, R2505Q, N2593S, S2621C	10 of 74 (13.5%)
11 to 99 CRAs	R216Q, P981R, V1125A, L1129S, T1160K, I1307K (3), E1317Q (4), V1352A, M1413V, C1578G, I1579V, D1714N, G1921S, P2158R, H2232D, A2274V, G2502S (5), R2505Q, I2573V, S2621C, A2795T	30 of 161 (18.6%)
≥100 CRAs	K150R, S643P, R653K, G2502S (3)	6 of 44 (13.6%)
Multiple CRAs (number unknown) ‡	K150R, E538V, P870S, C947S, P870S+M949I, L1129S, T1160K, I1307K, E1317Q (7), A1446T, K1454E, P1467S, A1474T, I1572T, P1934L, R2066G, I2329V, G2502S (10), I2541V, I2756V	35 of 201 (17.4%)
Total		81 of 480 (16.9%) † † $\chi^2 = 6.42$, $P = 0.0113$ N.B. 32 of 480 (6.7%)§ in the β-catenin down-regulating domain
Healthy controls		
(β-catenin down-regulating domain)		
	A1247T, I1307K (9), E1317Q (11), K1363I, M1413V (3), K1454E (2), P1458S, T1493M, P1584S, R1589G, R1589C, T1633K+T1655A+N1761T, R1676G, S1730F, Q1916K, I1975F	37 of 969 (3.8%)§ § $\chi^2 = 5.74$, $P = 0.0166$

NOTE: In total, 61 different rare nonsynonymous variants in *APC* were identified in this study. Each nonsynonymous variant was identified in a single patient/control within each group unless otherwise indicated in parentheses.

*To help define the phenotype of MAP, 3% of patients had ≤10 CRAs, 39% had 11 to 99 CRAs, 30% had ≥100 CRAs (one of which had severe polyposis; 1,000 CRAs), and 27% had multiple CRAs (number unknown).

† Comparison between numbers of non-FAP non-MAP and FAP/MAP patients carrying rare nonsynonymous variants (MAFs, <2%) within the *APC* ORF (N.B. significantly more non-FAP non-MAP patients also carried rare nonsynonymous variants compared with FAP patients alone; $\chi^2 = 5.50$, $P = 0.0191$).

‡ It should be noted that a large proportion of the non-FAP non-MAP patients had an undefined number of CRAs, and had this phenotype data been available, it may have altered the apparent prevalence of nonsynonymous variants in the 11 to 99 CRA group. However, the frequency of rare *APC* nonsynonymous variants in the undefined group was very similar to that found in the total group of non-FAP non-MAP patients with accurate CRA counts (17.4% versus 16.5%, respectively), suggesting that both groups had similar patient characteristics.

§Comparison between numbers of non-FAP non-MAP patients and healthy controls carrying rare nonsynonymous variants (MAFs, <2%) within the β-catenin down-regulating domain.

mutation were then sequenced for the ORF and splice sites of *MUTYH* to identify biallelic mutations. Peripheral blood DNA samples from healthy controls were sequenced over a ~2.4-kb region of *APC* spanning the β-catenin down-regulating domain by Agencourt Biosciences using six overlapping PCR fragments (primer sequences available upon request). Comparisons of numbers of patients harboring variants were performed using either the χ^2 test or Fisher's exact test.

β-Catenin–regulated transcription (CRT) assays. Variants were introduced into CMV-*APC* constructs using the QuikchangeXL site-directed mutagenesis kit (Stratagene). The luciferase reporter plasmids pTOPFLASH and pFOPFLASH, which contained wild-type or mutated TCF-response elements, were used to assess β-catenin–regulated transcription (CRT; ref. 10). SW480 cells were seeded at 10^4 cells per well in Optilux 96-well luminometer plates (VWR International) and,

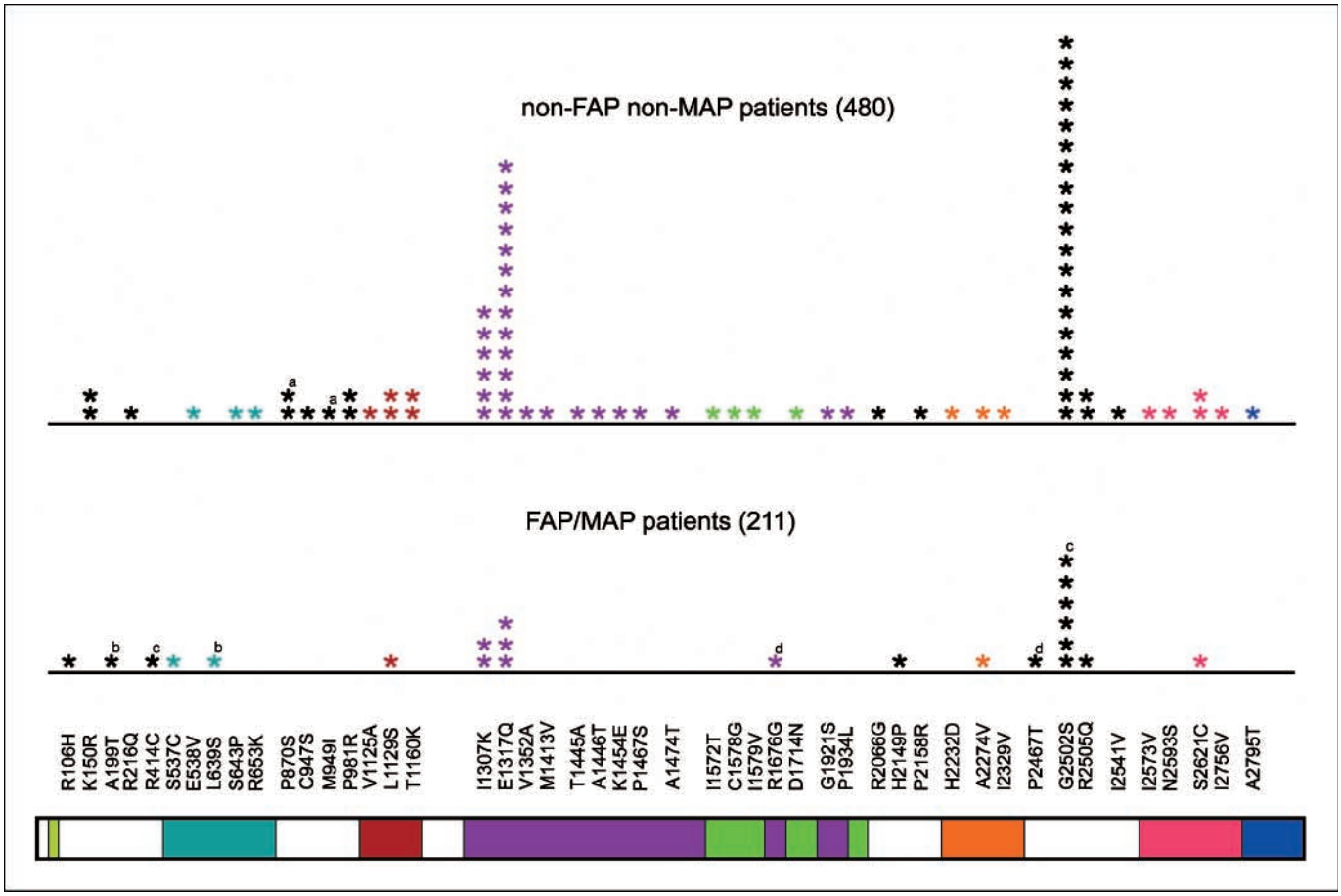

Figure 1. Distribution of inherited *APC* nonsynonymous variants in 480 non-FAP non-MAP patients with CRAs and 211 FAP/MAP patients. Ninety-seven patients carried a single variant and four patients (*a–d*) carried two variants. Functional domains (as described in refs. 4, 11–13) are colored: ■, oligomerization domain (codons 6–57); ■, armadillo region (codons 453–767); ■, 15–amino acid repeat β-catenin binding domain (codons 1020–1169); ■, 20–amino acid repeat β-catenin down-regulating domain (codons 1262–2033); ■, SAMP repeats/axin binding domain (codons 1562–2056); ■, basic domain (codons 2200–2400); ■, EB1 binding domain (codons 2559–2771); ■, HDLG binding domain (codons 2771–2843; domains not drawn to scale). Nonsynonymous variants are plotted as stars and colored according to the domains in which they lie; *black stars,* nonsynonymous variants that do not lie within known functional domains.

after 24 h, cotransfected with 25 ng of the reporter and *APC* variant plasmids and 2 ng of the transfection control plasmid pRL-SV40 (Promega). Assays were carried out using the Dual Luciferase Assay kit (Promega) at 24 h posttransfection. Luciferase readings were standardized for transfection efficiency, and results averaged from at least 10 independent experiments and compared using Students' *t* test.

***In silico* analyses.** Predictions using PolyPhen[5] were based on *Homo sapiens* APC (ENSG00000134982), and predictions using Align-Grantham Variation/Grantham Deviation (Align-GVGD)[6] were based on a multiple sequence alignment (created using T-coffee[7]) of APC orthologues from *Homo sapiens, Echinops telfairi* (ENSETEP00000013272), *Pan troglodytes* (ENSPTRP00000029327), *Macaca mulatta* (ENSMMUP00000000446), *Oryctolagus cuniculus* (ENSOCUP00000013423), *Bos taurus* (ENSBTAP00000025099), *Xenopus tropicalis* (ENSXETP00000046491), *Monodelphis domestica* (NSMODP00000016609), *Rattus norvegicus* (ENSRNOP00000027691), *Mus musculus* (ENSMUSP00000078337), *Dasypus novemcinctus* (ENSDNOP00000006189), and *Loxodonta africans* (ESLAFP00000011382).

[5] http://genetics.bwh.harvard.edu/pph/
[6] http://agvgd.iarc.fr/
[7] http://www.igs.cnrs-mrs.fr/Tcoffee/tcoffee_cgi/

Results and Discussion

We undertook comprehensive mutation analysis of the *APC* and *MUTYH* genes in 691 unrelated North American patients with CRAs. In total, we identified truncating mutations of *APC* in 178 patients and biallelic mutations of *MUTYH* in 33 patients, confirming FAP and MAP, respectively. Among these 211 FAP/MAP patients, 3.3% had ≤10 CRAs, 20.9% had 11 to 99 CRAs, 46% had ≥100 CRAs, and 29.9% had multiple CRA (number unknown), as recorded at colonoscopy or colectomy. Among the 480 non-FAP non-MAP patients, 15.4% had ≤10 CRAs, 33.5% had 11 to 99 CRAs, 9.2% had ≥100 CRAs, and 41.9% had multiple CRA (number unknown).

Nonsynonymous variants in patients with CRAs. In total, 48 different rare [minor allele frequencies (MAF), <2%] nonsynonymous variants were identified spanning the *APC* ORF in 101 patients (14.6%); 97 patients carried single heterozygous variants, and four patients carried two heterozygous variants (three FAP patients carried A199T and L639S, R414C and G2502S, and R1676G and P2467T, respectively, and one non-FAP non-MAP patient carried P870S and M949I). Only 20 of the 211 FAP/MAP patients (9.5%) carried rare nonsynonymous variants. In contrast, 81 of the

480 patients (16.9%) without FAP or MAP carried such variants (χ^2 = 6.42, P = 0.0113), suggesting that some nonsynonymous variants in *APC* might predispose to CRAs (Table 1; Fig. 1; functional domains are described in refs. 4, 11–13). When non-FAP non-MAP patients were classified according to the number of CRAs, the group with 11 to 99 CRAs had a higher frequency of rare nonsynonymous variants (18.6% of patients) compared with the groups with ≤10 CRAs (13.5% of patients) or ≥100 CRAs (13.6% of patients; Table 1). Indeed, significantly more non-FAP non-MAP patients with 11 to 99 CRAs carried rare nonsynonymous variants compared with the FAP/MAP patients (30 of 161 versus 20 of 211, χ^2 = 6.579, P = 0.0103).

We considered whether the overrepresentation of rare nonsynonymous variants in the non-FAP non-MAP patients was simply due to an overrepresentation of the previously studied variants I1307K and E1317Q. We found that similar proportions of non-FAP non-MAP and FAP/MAP patients carried I1307K (6 of 480 versus 2 of 211, P = 0.538). Although we did find that more non-FAP non-MAP patients carried E1317Q than FAP/MAP patients [13 of 480 (2.7%) versus 3 of 211 (1.4%)], this was not significant (P = 0.229). Importantly, when we only considered alleles with MAFs of <0.5% [thereby excluding G2502S (1.74%), E1317Q (1.16%), and I1307K (0.58%)], we still found that more non-FAP non-MAP patients carried rare nonsynonymous variants compared with FAP/MAP patients (43 of 480 versus 10 of 211, χ^2 = 3.68; P = 0.0549) and observed a particular strong overrepresentation in the non-FAP non-MAP cases with 11 to 99 CRAs (18 of 161 versus 10 of 211, χ^2 = 5.443; P = 0.0197). In contrast, similar proportions of non-FAP non-MAP and FAP/MAP patients carried rare (MAFs, <2%) synonymous variants, and the frequencies of seven common polymorphisms were almost identical between the two groups (Table 2). Both groups also had similar self-reported ethnic backgrounds (Supplementary Table S1). It was therefore highly unlikely that these findings could be attributed to population stratification.

Nonsynonymous variants in healthy controls. *APC* encodes a large protein which plays a role in signal transduction in the wnt signaling pathway, and it has been proposed that a specific level of β-catenin signaling, mediated by selection for *APC* genotypes that retain some 20–amino acid β-catenin down-regulating repeats (20AAR), is required for colorectal tumor formation (14). We found that significantly more non-FAP non-MAP patients had rare nonsynonymous variants in the β-catenin down-regulating domain (spanning codons 1262–2033) compared with FAP/MAP patients (32 of 480 versus 6 of 211, χ^2 = 4.12, P = 0.0423). Using a sequencing approach, we sought rare nonsynonymous variants in the ~2.4-kb β-catenin down-regulating domain in 969 unrelated North American healthy controls that were matched to the unrelated North American non-FAP non-MAP patients for age, sex, and race. In total, 18 different rare nonsynonymous variants were identified in the β-catenin down-regulating domain in 37 healthy controls; 36 controls carried single heterozygous variants, and one carried three heterozygous variants (T1633K, T1655A, and N1761T; Table 1). Five of 18 of the nonsynonymous variants (I1307K, E1317Q, M1413V, K1454E, and R1676G) were previously identified in the patient cohort, whereas the remaining 13 variants were unique to the control group.

We found that significantly more non-FAP non-MAP patients carried rare nonsynonymous variants in the β-catenin down-regulating domain compared with the healthy controls (32 of 480 versus 37 of 969, χ^2 = 5.74, P = 0.0166), and this overrepresentation was highest in the non-FAP non-MAP patients with 11 to 99 CRAs (13 of 161 versus 37 of 969, χ^2 = 5.914, P = 0.0150; Table 1). In terms of individual variants, we did not observe an overrepresentation of I1307K in the non-FAP non-MAP patients versus controls (6 of 480 versus 9 of 969, respectively). However, we did find that significantly more non-FAP non-MAP patients carried E1317Q compared with healthy controls (13 of 480 versus 11 of 969, χ^2 = 4.88, P = 0.0272), although, even when this variant was excluded from the analyses, significantly more non-FAP non-MAP patients with 11 to 99 CRAs carried other rare nonsynonymous variants in the β-catenin down-regulating domain compared with controls (9 of 161 versus 26 of 969, χ^2 = 3.887, P = 0.0487). In contrast, the frequencies of rare synonymous variants in this region and common polymorphisms were almost identical between the non-FAP non-MAP patients and controls (Table 2). These data suggest that a proportion of nonsynonymous variants in the β-catenin down-regulating domain of *APC* are likely to alter β-catenin signaling to promote tumorigenesis. Given that we sequenced only ~27% of the *APC* ORF in the healthy controls (due to cost considerations) and because nonsynonymous variants are spread

Table 2. Frequency of rare *APC* synonymous variants and common *APC* polymorphisms in patients and healthy controls

	FAP/MAP patients (211)	Non-FAP non-MAP patients (480)	Healthy controls (969)
Rare synonymous variants			
APC ORF	16.1%	14.8%	N/A
β-Catenin down-regulating domain	6.2%	6.0%	5.7%
Common polymorphisms			
D1822V	24.1%	22.8%	21.1%
Y486 (1458C>T)	45.5%	44.4%	N/A
A545 (1635A>G)	41.2%	39.7%	N/A
T1493 (4479A>G)	41.0%	39.8%	38.4%
G1678 (5034A>G)	40.5%	39.2%	38.1%
S1756 (5268G>T)	41.2%	39.2%	38.3%
P1960 (5880A>G)	41.0%	39.8%	38.0%

NOTE: Twenty-nine different rare (MAFs, <2%) synonymous variants were found spanning the *APC* ORF in 105 patients (15.2%); 95 of these carried a single variant (93 in a heterozygous state), nine carried two heterozygous variants, and one carried three heterozygous variants. Eleven different rare synonymous variants were found spanning the β-catenin down-regulating domain in 55 healthy controls (5.7%). N/A, not assessed.

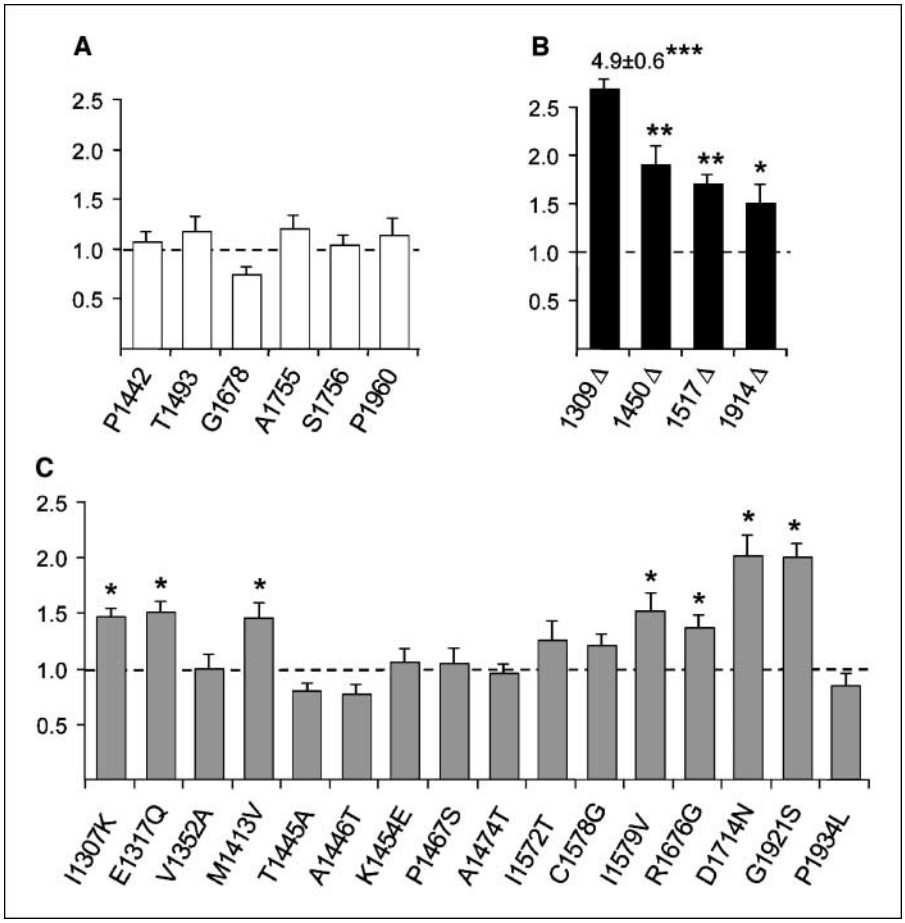

Figure 2. Functional analysis of synonymous, truncating, and nonsynonymous variants in the β-catenin down-regulating domain of *APC*. All six synonymous variants suppressed CRT as effectively as wild type (*A*), whereas all four truncating mutations failed to suppress CRT (*B*); *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$. *C,* seven of 16 nonsynonymous variants (43.8%) also failed to suppress CRT (*$P < 0.05$). *Dashed line,* the normalization of values to wild-type APC (1.0 relative light unit).

throughout the gene (Fig. 1), it is likely that variants in other functional domains also play a role in tumorigenesis.

Functional analyses of nonsynonymous variants in the β-catenin down-regulating domain. We examined the functional consequences of 16 of the nonsynonymous variants identified within the β-catenin down-regulating domain using CRT assays. First, to confirm the ability of the assay system to detect clinically significant effects on β-catenin–associated signaling, we made and tested six synonymous variants (normal controls) and four truncating mutations (mutant controls) within the β-catenin down-regulating domain. The four truncating mutations 1309Δ, 1450Δ, 1517Δ, and 1914Δ were predicted to retain one, two, three, and five 20AARs, respectively. As expected, the six synonymous variants and the wild-type *APC* construct all suppressed CRT in an *APC*-deficient cell line (Fig. 2*A*). Consistent with the notion that mutations resulting in the retention of different numbers of 20AARs lead to different levels of β-catenin–associated signaling, 1309Δ, which is associated with a severe FAP phenotype, failed to suppress CRT ($P < 0.0001$) whereas 1450Δ, 1517Δ, and 1914Δ suppressed CRT more effectively than 1309Δ but not as effectively as the wild-type construct ($P < 0.05$; Fig. 2*B*). There was a clear relationship between the predicted number of 20AARs retained and CRT activity ($7>5>3>2 \gg 1$). Next, we tested the 16 nonsynonymous variants, and in accordance with our hypothesis that some of these rare variants may compromise function, seven (43.8%) had a significantly reduced ability to suppress CRT (Fig. 2*C*). Both I1307K and E1317Q were found to be functionally compromised.

***In silico* predictions of likely pathogenicity.** Other nonsynonymous variants could interfere with APC's roles in mediation of intercellular adhesion, stabilization of the cytoskeleton, chromosome stability or regulation of the cell cycle, and apoptosis (4, 15). We used the programs PolyPhen and Align-GVGD to help predict the functional consequences of all 61 different nonsynonymous variants that we identified in *APC*. *In silico* analyses using PolyPhen predicted that 24 nonsynonymous variants (39%) were likely to be damaging, whereas Align-GVGD predicted that 28 variants (46%), 17 of which were also called damaging by PolyPhen, were likely to alter function (Supplementary Table S2).

Conclusions

Although some studies have already suggested that the nonsynonymous variants I1307K and E1317Q predispose to CRAs and CRCs (7–9, 16, 17), other studies have not supported these observations (18, 19). Here, we show that significantly more non-FAP non-MAP patients with CRAs carry E1317Q compared with controls, clearly supporting a role for this variant in predisposing to CRAs. Although we did not find a similar overrepresentation of I1307K, this variant was less frequently observed; it is normally found in 6–7% of the Ashkenazi Jewish population (16), but <4% of our patients were from this ethnic background. Importantly, our study showed that even when I1307K and E1317Q were excluded, significantly more non-FAP non-MAP patients carried a variety of other rare nonsynonymous variants, indicating that numerous rare variants in *APC* act as low penetrance disease alleles.

Other investigators (20) have recently reported that rare variants in the Wnt signaling genes *AXIN1* and *CTNNB1* and the mismatch repair genes *hMLH1* and *hMSH2*, together with E1317Q in *APC*, are overrepresented in patients with multiple CRAs. Here, we present genetic, functional, and *in silico* data to show that individually rare, but collectively common, inherited nonsynonymous variants in *APC* also play a significant role in multifactorial inherited predisposition to CRAs, with particular importance in patients with a "moderate" (11 to 99 CRA) polyposis phenotype. Further characterization of these and other low penetrance alleles should help contribute to the future goal of personalized medicine.

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Conflict of interest: J.P. Cheadle and J.R. Sampson have previously made intellectual property rights applications in relation to *MUTYH* sequence variants, which are licensed to Myriad Genetic Laboratories, Inc.

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