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Inactivation of Germline Mutant *APC* Alleles by Attenuated Somatic Mutations: A Molecular Genetic Mechanism for Attenuated Familial Adenomatous Polyposis

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Germline mutations of the adenomatous polyposis coli (*APC*) tumor-suppressor gene result in familial adenomatous polyposis (FAP). Patients with FAP typically develop hundreds to thousands of benign colorectal tumors and early-onset colorectal cancer. A subset of germline *APC* mutations results in an attenuated FAP (AFAP) phenotype, in which patients develop fewer tumors and develop them at an older age. Although a genotype-phenotype correlation between the locations of *APC* germline mutations and the development of AFAP has been well documented, the mechanism for AFAP has not been well defined. We investigated the mechanism for AFAP in patients carrying a mutant *APC* allele (APC_{AS9}) that has a mutation in the alternatively spliced region of exon 9. APC_{AS9} was found to down-regulate β-catenin–regulated transcription, the major tumor-suppressor function of *APC*, as did the wild-type *APC*. Mutation analysis showed that both APC_{AS9} and the wild-type *APC* alleles were somatically mutated in most colorectal tumors from these patients. Functional analysis showed that 4666insA, a common somatic mutation in APC_{AS9} in these tumors, did not inactivate the wild-type *APC*. Our results indicate that carriers of APC_{AS9} develop fewer colorectal tumors than do typical patients with FAP because somatic inactivation of both *APC* alleles is necessary for colorectal tumorigenesis. However, these patients develop colorectal tumors more frequently than does the general population because APC_{AS9} is inactivated by mutations that do not inactivate the wild-type *APC*.

Introduction

Familial adenomatous polyposis (FAP [MIM 175100]) is an autosomal dominant inherited disease that predisposes patients to colorectal cancer. Patients with FAP typically develop hundreds to thousands of colorectal adenomas in their 2d or 3d decades of life. If not surgically removed, some of these benign adenomas will progress to malignant carcinoma. FAP is caused by germline mutation of the adenomatous polyposis coli (*APC*) tumor-suppressor gene (Groden et al. 1991; Joslyn et al. 1991; Kinzler et al. 1991; Nishisho et al. 1991). Somatic mutations in *APC* also occur in most sporadic colorectal tumors (Miyoshi et al. 1992; Powell et al. 1992; Miyaki et al. 1994). Consistent with Knudson's two-hit hypothesis that both alleles of a tumor-suppressor gene are mutated in a tumor (Knudson 1985), mutation of both *APC* alleles has been demonstrated in FAP-related and sporadic colorectal tumors (Miyoshi et al. 1992; Powell et al. 1992; Miyaki et al. 1994). The formation of most colorectal tumors is believed to be initiated by the mutation of both *APC* alleles (Kinzler and Vogelstein 1996).

APC expresses a major mRNA of ~10.5 kb that encodes a protein of 2,843 amino acids (Groden et al. 1991; Joslyn et al. 1991; Kinzler et al. 1991). *APC* exon 15 contains 6.5 kb of the 3′ coding region and includes a mutation-cluster region in which most somatic mutations occur (Groden et al. 1991; Joslyn et al. 1991; Miyoshi et al. 1992; Miyaki et al. 1994). The tumor-suppressor function of *APC* is believed to involve the regulation of β-catenin–regulated transcription (CRT [Korinek et al. 1997; Morin et al. 1997; Polakis 1999; Peifer and Polakis 2000]). β-Catenin can associate with the Tcf/Lef family of DNA-binding proteins and can form DNA sequence–specific transcription complexes (Behrens et al. 1996; Molenaar et al. 1996; Korinek et al. 1997). APC regulates CRT by associating with β-catenin, together with glycogen synthase kinase-3β (GSK3β) and axin or conductin, and promoting its degradation (Munemitsu et al. 1995; Rubinfeld et al. 1996; Behrens et al. 1998; Hart et al. 1998; Polakis 1999; Peifer and Polakis 2000). The identification of human and rat colorectal tumors that do not have inactivating

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mutations in *APC* but instead express β-catenin mutants resistant to *APC*-mediated regulation strongly supports the hypothesis that APC exerts its tumor-suppressor function by regulation of CRT (Morin et al. 1997; Dashwood et al. 1998; Sparks et al. 1998; Takahashi et al. 1998).

In addition to the major mRNA, *APC* also expresses several minor alternatively spliced mRNA (Groden et al. 1991; Samowitz et al. 1995). One alternatively spliced *APC* mRNA, *APCdA9,* lacks 303 nucleotides of the alternatively spliced region of exon 9 (Groden et al. 1991). The APCdA9 protein encoded by *APCdA9* contains all domains known to be necessary for regulation of CRT (Groden et al. 1991; Munemitsu et al. 1995), although this function could be impaired by altered three-dimensional structure.

Patients with a form of FAP called "attenuated FAP" (AFAP) develop fewer colorectal tumors, and at older ages, than do patients with typical FAP (Lynch et al. 1992, 1995). Mounting evidence indicates that individuals carrying an APC_{AFAP} allele usually develop AFAP (Spirio et al. 1993; van der Luijt et al. 1995, 1996; Friedl et al. 1996; Soravia et al. 1998), although there could be significant variation in the phenotype of individuals carrying the same germline mutation (Brensinger et al. 1998; Soravia et al. 1998). APC_{AFAP} includes *APC* alleles with a germline mutation in the first four coding exons ($APC_{1–4}$), in the alternatively spliced region of exon 9 (APC_{AS9}), or in the 3′ half of the coding region (APC_{3H}; for descriptions of alleles, see the Appendix).

There are two alternative genetic explanations for how patients carrying APC_{AFAP} develop AFAP, according to Knudson's two-hit model. One is that APC_{AFAP} does not have tumor-suppressor activity and that a single somatic mutation inactivating the wild-type *APC* allele will lead to colorectal tumorigenesis. This is similar to what happens in patients with FAP and could explain why patients with AFAP are susceptible to development of colorectal tumors. However, for patients with AFAP to have the attenuated phenotype, inactivation of the wild-type *APC* allele must either occur less frequently than in typical FAP or not be sufficient for tumorigenesis. Alternatively, the APC_{AFAP} alleles still have tumor-suppressor activity, and somatic inactivation of both *APC* alleles is necessary for tumor formation. This resembles the development of sporadic colorectal tumors in people without a germline *APC* mutation and could explain why patients with AFAP have the attenuated phenotype. However, *APC* must be inactivated more readily in patients with AFAP than in the general population, because patients with AFAP are still predisposed to colorectal tumorigenesis.

A study of colorectal tumors from patients carrying $APC_{1–4}$ demonstrated that some of these tumors had two somatic mutations (Spirio et al. 1998). A specific *APC* mutation, 4666insA, was found frequently in these tumors and was found to occur at the $APC_{1–4}$ allele. It was proposed that, for tumors to form in these patients, the wild-type *APC* allele must be somatically inactivated first and that then the germline mutant allele must be somatically inactivated by either small mutations or loss of heterozygosity (LOH). However, it remained unclear why these patients were predisposed to colorectal tumorigenesis. The present report describes our investigation of the underlying mechanism of AFAP in patients carrying APC_{AS9}.

Patients, Material, and Methods

Patients and Tumor Samples

The two patients were from a family carrying a germline nonsense mutation at *APC* codon 332 (CGA→TGA; data not shown). Patient 1 (a 47-year-old male) had 30 colorectal adenomas <2 mm and a history of 2 1-cm adenomas that had been previously excised. Patient 2 (a 15-year-old male) had three colorectal adenomas <1 mm. Neither patient had a history of cancer or surgery. Informed consent for collection of tumor samples was obtained from patients, and the research was approved by the M.D. Anderson Cancer Center's institutional review board.

The adenomas were separated from adjacent normal tissues by microdissection of fresh biopsy samples. Single crypts were isolated from fresh adenoma biopsies by a modification of a method described elsewhere (Cheng et al. 1984). In brief, fresh mucosal biopsy samples were incubated at 37°C for 10 min in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution containing 30 mM EDTA, then were vibrated gently to separate the epithelium from the stroma. The isolated crypts were then incubated in a buffer containing $CaCl_2$ and $MgCl_2$, to restore normal chromatin structure and crypt architecture. Finally, the crypts were fixed first in 10% buffered formalin for 2 h, then in 70% ethanol overnight. Confocal microscopy of propidium iodide–stained single crypts confirmed the complete isolation of the epithelial crypt cells from the surrounding mucosa.

Mutation Detection

Genomic DNA was isolated from individual crypts by proteinase K digestion, followed by phenol-chloroform extraction and ethanol precipitation. Mutations of *APC* were detected using the in vitro synthesized-protein assay (IVSP), followed by DNA sequencing, as described elsewhere (Powell et al. 1993; Su et al. 2000). All tumors were analyzed for codons 686–1686, and tumors with only one somatic mutation in this region were then analyzed for codons 1554–2843. Templates for IVSP were

generated by nested PCR. Primers used for the first-step PCR were 5′-CAAATCCTAAGAGAGAACAAC-3′ and 5′-GACGCAGATGCTTGCTGG-3′ for codons 686–1686 and 5′-GATTTTCTTGTTCATCCAGCC-3′ and 5′-GAGTGGATCCCAAAATAAGACC-3′ for codons 1554–2843. PCR was performed using the Expand Long Template PCR System (Boehringer-Mannheim). One tenth of the genomic DNA obtained from a single crypt was used in the first-step PCR. The reaction consisted of 1 cycle of 2 min at 92°C; 20 cycles each of 15 s at 92°C, 1 min at 55°C, and 2.5 min at 68°C; and 1 cycle of 5 min at 68°C. One microliter of the first-step amplification product was used in the second-step PCR. The PCR and the coupled in vitro transcription and translation reactions were performed as described elsewhere (Su et al. 2000).

Plasmids

The reporter plasmids for assessment of CRT, pTOPFLASH and pFOPFLASH were gifts from Dr. H. Clevers (University Hospital, the Netherlands) and carry the luciferase gene regulated by wild-type and mutant T cell factor–binding motifs, respectively (Korinek et al. 1997). The control reporter pcDNA3Luc was generated by insertiing the coding region of luciferase downstream of the cytomegaloviral early promoter in the plasmid pcDNA3 and was a gift from Drs. M.-C. Hung and D.-H. Yan (M.D. Anderson Cancer Center). The plasmid pBI-MCS-EGFP (Yu et al. 1999) and plasmids containing the wild-type *APC* and *APC1309* and were gifts from Drs. K.W. Kinzler and B. Vogelstein (Johns Hopkins University). The plasmid pUHD15-1 (Gossen and Bujard 1992) was provided by Dr. H. Bujard (Universität Heidelberg), and pRL-TK was purchased from Promega.

APCdA9 was generated by replacement of a fragment of the full-length *APC* cDNA, containing the entire exon 9, by a corresponding fragment that did not have the alternatively spliced region of exon 9 obtained by reverse transcription–PCR. *APC1556* was generated by replacement of a fragment of wild-type *APC* containing codon 1556 by a corresponding PCR fragment containing an A inserted at codon 1556. Fragments that replaced the wild-type *APC* were completely sequenced, to ensure that there were no unintended sequence alterations. *APCdA,1556* was generated by replacement of the 5′ 2.2-kb *Bam*HI-*Stu*I fragment of *APC1556* by the corresponding fragment from *APCdA9*.

Three different expression vectors, pCMV-NEO-BAM (Baker et al. 1990), pCIN, and pTBI, were used to express *APC*. To generate pCIN, the *Sal*I-*Bam*HI fragment containing rabbit β-globin intron 2 and part of its flanking exons was isolated from pCMV-NEO-BAM, the *Sal*I site was filled in, and the fragment was inserted between the *Bam*HI and the filled-in *Hin*dIII site of pcDNA3 (Invitrogen). To generate pTBI, the *Hin*dIII-*Xho*I fragment containing β-globin intron was isolated from pCIN, the *Hin*dIII site was filled in, and the fragment was inserted between the *Pvu*II and *Xho*I sites of pBI-MCS-EGFP. *APC* cDNAs were inserted at the *Bam*HI site of pCIN and pCMV-NEO-BAM and at the *Bgl*II site of pTBI.

Cell Lines

All cell lines were obtained from American Type Culture Collection. MCF7 is a human breast cancer cell line. SW480, DLD-1, and HCT116 are human colon cancer cell lines. SW480 and DLD-1 express the wild-type β-catenin that can be regulated by APC, whereas HCT116 expresses a mutant β-catenin that is resistant to APC regulation (Morin et al. 1997).

Results

We first determined whether *APCdA9* had tumor-suppressor activity. The alternatively spliced *APCdA9* transcript expressed from APC_{AS9} does not contain the germline mutation and is expected to express the same APCdA9 protein that is expressed from the wild-type *APC*. Thus, APC_{AS9} would have tumor-suppressor activity if *APCdA9* has tumor-suppressor activity. We compared the ability of *APCdA9* to regulate CRT with that of the wild-type *APC* and of the mutant *APC1309*, a common *APC* mutant deficient in regulation of CRT (Nagase and Nakamura 1993; Miyaki et al. 1994; Morin et al. 1997). As it did in previous reports of the wild-type *APC* (Korinek et al. 1997; Morin et al. 1997), *APCdA9* reduced the expression of a reporter controlled by CRT (pTOPFLASH) but did not significantly affect the expression of reporters that were not controlled by CRT (pcDNA3Luc and pFOPFLASH; fig. 1). These results suggested that APC_{AS9} had tumor-suppressor activity. However, because of the normally low expression level of *APCdA9* (Groden et al. 1991), it was not clear whether APC_{AS9} needed to be somatically mutated for colorectal tumors to form in APC_{AS9} carriers.

We next examined whether APC_{AS9} was somatically mutated in colorectal tumors from patients carrying APC_{AS9}. Nine early colorectal adenomas in two patients from a family carrying APC_{AS9} were examined for somatic mutations in *APC* exon 15. Single crypts were isolated from each tumor, and genomic DNA was prepared from each crypt, for use in the analysis. All nine tumors had somatic mutations, including one that had LOH at the *APC* locus, and five of them had somatic mutations in both *APC* alleles (fig. 2 and table 1). DNA sequencing of *APC* exon 9 of the tumor having loss of one *APC* allele showed that the allele lost was APC_{AS9}

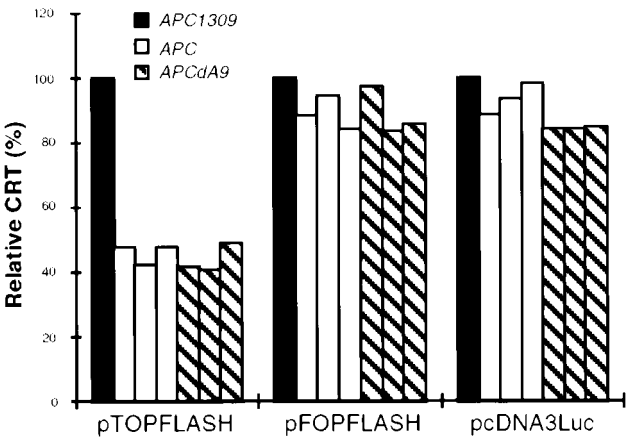

Figure 1 Regulation of CRT by *APCdA9*. SW480 cells were transfected with the combination of pCMVβgal, a reporter plasmid (pTOPFLASH, pFOPFLASH, or pcDNA3Luc), and an *APC* expression vector, as indicated. Results of three separate experiments using pCIN to express *APC* are shown. The activities of the wild-type *APC* and *APCdA9* in the regulation of CRT are compared with that of *APC1309*. Similar results were obtained using pCMV-NEO-BAM to express *APC* (data not shown).

(data not shown). Four of these nine tumors had the 4666insA mutation (table 1). To determine which *APC* allele had 4666insA, a DNA fragment containing this mutation and two intragenic polymorphic nucleotides, 4479 and 5034 (Nagase et al. 1992; Powell et al. 1992), was amplified by PCR, cloned, and sequenced. The allelotype of the wild-type *APC* allele, with regard to these two polymorphisms, was determined by sequencing of a similar PCR product amplified using DNA from the tumor that had lost the APC_{AS9} allele. The results showed that 4666insA occurred in APC_{AS9} in all three tumors from patient 1 (data not shown). The allele carrying this mutation in the tumor from patient 2 could not be determined because he was homozygous for these two polymorphic nucleotides. The allele carrying 4616delAG in a tumor from patient 1 also was found to be APC_{AS9} (data not shown). Therefore, somatic mutation of the APC_{AS9} allele could be demonstrated in eight of the nine tumors examined, including three of the four tumors that had only one somatic *APC* mutation identified.

The high frequency of the 4666insA mutation in these tumors—and, specifically, in the APC_{AS9} allele—suggested that, although this mutation could inactivate APC_{AS9}, it might not significantly affect the function of wild-type *APC*. To test this hypothesis, the 4666insA mutation was introduced into *APC* and *APCdA9*, to generate *APC1556* and *APCdA,1556*, respectively. Immunoblot analysis confirmed that this mutation resulted in the truncation of APC protein (fig. 3*A*). The ability of these forms of *APC* to down-regulate CRT was then examined. As expected, *APC* and *APCdA9* effectively regulate CRT in both SW480 and DLD-1 cells but not in HCT116 cells (fig. 3*B*). The 4666insA mutation did not appear to affect the activity of *APC* and *APCdA9* in SW480 cells. This mutation reduced, but did not abolish, their activity in DLD-1 cells. Consistent with its ability to regulate CRT, APC1556 was able to reduce the β-catenin–protein level in SW480 cells (fig. 3*C*). These results showed that the 4666insA mutation did not abolish the function of *APC* or *APCdA9*.

Discussion

We have provided evidence that APC_{AS9} has tumor-suppressor activity (i.e., regulation of CRT) and that it is somatically mutated in colorectal tumors in APC_{AS9} carriers. Somatic mutations at the APC_{AS9} allele were identified in eight of the nine tumors examined. This finding supports the premise that all nine tumors had somatic mutations in both *APC* alleles, with some of these mutations probably in *APC* regions that we did not examine. The absence of the wild-type IVSP product in those single crypts having two somatic mutations strongly supports the possibility that both somatic mutations occurred in the same cell (fig. 2). Because all tumors examined were <2 mm in diameter, somatic inactivation of both *APC* alleles was most likely to be necessary prior to colorectal tumorigenesis in these patients. This would explain why patients carrying APC_{AS9} develop fewer tumors, and at older ages, than do patients with typical FAP.

Somatic mutations of both the wild-type and the germline mutant *APC* alleles was reported for some colorectal tumors from patients with AFAP who carry APC_{1-4} (Spirio et al. 1998). However, the rate of double somatic mutations is significantly higher in tumors with

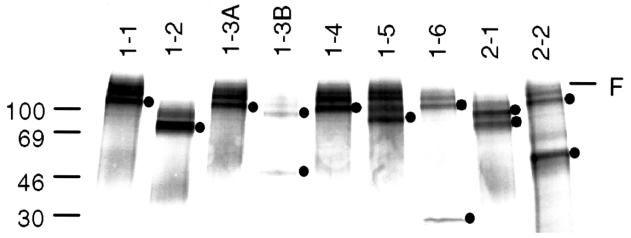

Figure 2 Detection of somatic *APC* mutation. The result of IVSP analysis of codons 686–1686 of a representative single crypt from each tumor is shown. The use of single isolated crypts in this assay allowed the easy detection of LOH in tumor 1-2. "F" indicates the position of the full-length product presented in tumors 1-1, 1-3A, 1-4, and 1-5. Dots indicate mutant products. The positions of the molecular-weight standards are shown on the left.

Table 1

Somatic *APC* Mutations

Patient tumor	Somatic Mutation(s)	Codon(s) Affected	No. of crypts	
			Examined	With Mutation
1-1	4666insA[a]	1556	5	5
1-2	3896del11, LOH[b]	1298–1301	11	11
1-3A	4666insA[a]	1556	12[c]	4
1-3B	3158delA, 4392delAG	1053, 1465–1466	12[c]	8
1-4	4616delAG[a]	1539–1540	5	3[d]
1-5	4216delAG	1406	5	5
1-6	2562delGA, 4666insA[a]	855, 1556	5	5
2-1	3925del5, R1450X	1309–1310, 1450	7	6[e]
2-2	R1114X, 4666insA	1114, 1556	7	6[d]

[a] In APC_{AS9} allele.
[b] Lost allele was the APC_{AS9} allele.
[c] Isolated from what appeared to be one adenoma. The different mutation patterns suggest that either the adenoma was heterogeneous or two adjacent adenomas were confluent.
[d] Remaining crypts did not have mutations.
[e] Remaining crypt had only 3925del5.

APC_{AS9}, which we examined, than in those with $APC_{1–4}$, which have been reported elsewhere (5/9 vs. 7/35 [Spirio et al. 1998]; $P < .05$, Fisher's exact test). The proportion of tumors having LOH as one of the double somatic mutations is significantly lower in tumors with APC_{AS9} than that reported in tumors with $APC_{1–4}$ (1/5 vs. 6/7 [Spirio et al. 1998]; $P < .05$), although the overall rates of LOH in these two groups of tumors are comparable (1/9 vs. 8/66 [Spirio et al. 1998]; $P = .7$) and are similar to that reported for FAP-associated adenomas (Miyaki et al. 1994). These differences could reflect the biological differences between these two mutant *APC* alleles. The difference also may be partly due to the greater sensitivity of our method, which analyzes single crypts, for detection of mutations. A drawback of both studies is that each examined tumors from patients in a single family. Additional investigations of tumors from other families should provide more-conclusive information.

Patients with AFAP who carry APC_{AS9} are predisposed to colorectal tumorigenesis despite their attenuated phenotype. Therefore, the *APC* gene in these patients must be somatically mutated more easily than it is in the general population. One possibility is that germline mutations increase the mutation rate of APC_{AS9}. Increased somatic mutation frequency has been demonstrated in the *APCI1307K* allele, and this may explain why *APCI1307K* carriers have an increased risk of colon cancer (Laken et al. 1997; Gryfe et al. 1998, 1999). Although the possibility that APC_{AS9} alleles have an increased mutation rate cannot be completely ruled out, we do not have evidence for it. Instead, we propose that APC_{AS9} alleles are inactivated more easily because they can be inactivated by mutations that do not inactivate wild-type *APC*. The 4666insA mutation is common in tumors from patients with AFAP who carry either APC_{AS9} or $APC_{1–4}$ (Spirio et al. 1998; present study). In contrast, this mutation was rarely found in sporadic colorectal tumors or tumors from patients with typical FAP (Powell et al. 1992; Miyaki et al. 1994). Although the 4666insA mutation occurs at a mononucleotide repeat, it is not caused by increased microsatellite instability in tumors from patients with AFAP (Spirio et al. 1998). We suggest that the 4666insA mutation may occur frequently because it arises within a stretch of six As and that it is rarely found in non–AFAP-associated colorectal tumors because it only slightly impairs the tumor-suppressor function of the wild-type *APC*. However, this apparently weak mutation may be able to inactivate the residual activity of APC_{AS9} because of the normally low level of *APCdA9* (fig. 4). Additional studies of tumors from patients with AFAP who carry different germline *APC* mutations will be needed to provide additional support for this hypothesis.

APC1556 was found to down-regulate CRT, although it lacks any conductin-binding domains and therefore is unlikely to interact with axin or conductin directly (Behrens et al. 1998). However, APC1556 contains all three 15-amino-acid– and three of seven 20-amino-acid–repeat sequences that interact with β-catenin (Su et al. 1993; Munemitsu et al. 1995). Because axin binds β-catenin and GSK3β directly (Behrens et al. 1998; Hart et al. 1998), APC1556 could associate with β-catenin/axin/GSK3β complex and promote the degradation of β-catenin, albeit less efficiently than do APC proteins that can bind axin directly. This hypothesis is consistent with results of a study comparing the CRT activity in mouse embryonic stem cells expressing different Apc mutants (Smits et al. 1999).

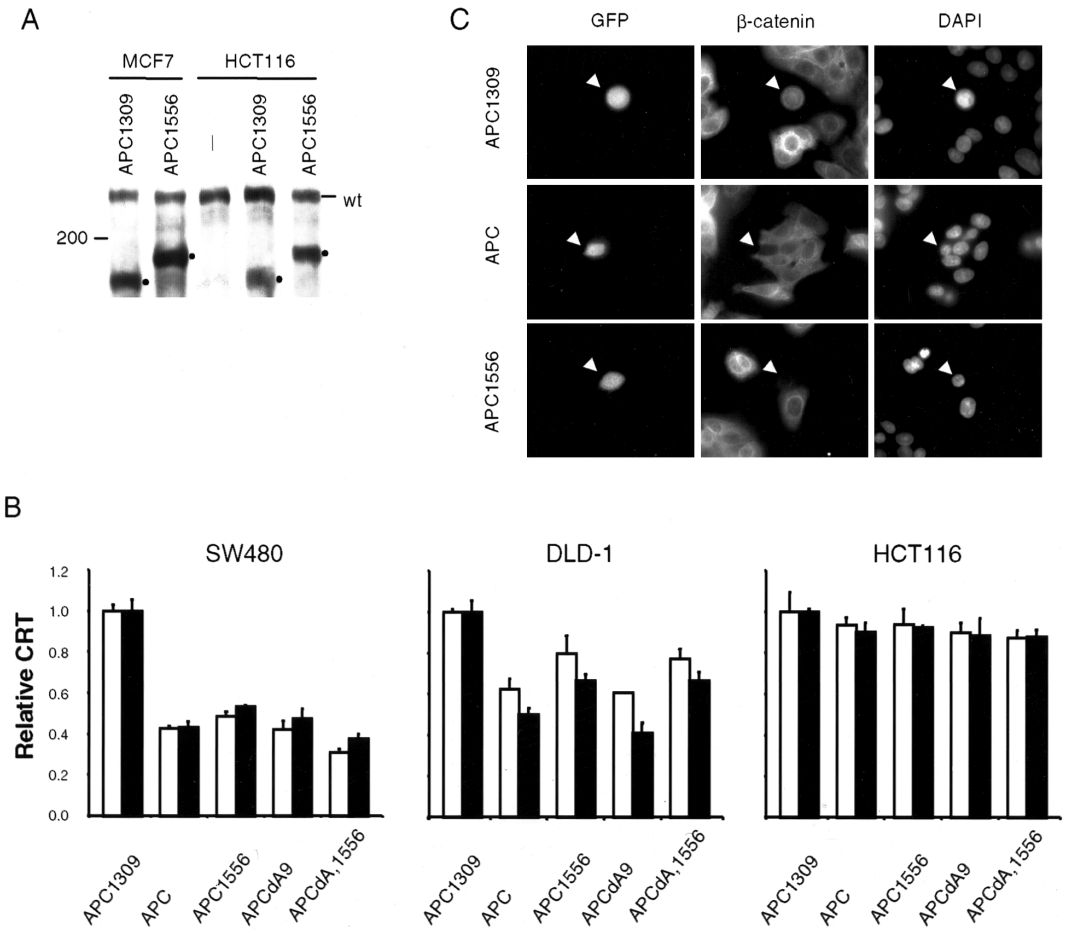

Figure 3 Regulation of β-catenin by APC1556. *A,* Expression of APC1556. MCF7 and HCT116 cell lines were transfected with pCIN alone (—) or pCIN expressing the indicated mutant *APC.* APC proteins were detected by immunoblotting. "wt" indicates the endogenous wild-type APC. Dots indicate mutant APC proteins. *B,* Regulation of CRT by APC1556. SW480, DLD-1, and HCT116 cell lines were transfected with a combination of pRL-TK, pTOPFLASH, and an APC expression vector, as indicated. The unblackened and blackened bars indicate expression of APC by pCIN and by pCMV-NEO-BAM, respectively. The mean and SD of the result of triplicate experiments are shown. The activities of the wild-type *APC, APC1556, APCdA9,* and *APCdA,1556* in the regulation of CRT are compared with that of *APC1309.* (*C*) Reduction of β-catenin by APC1556. SW480 cells were transfected with pUHD15-1 and pTBI, expressing indicated APC. Cells were immunostained with a monoclonal antibody against β-catenin. Transfected cells were identified by their expression of green fluorescence protein (GFP), because pTBI also expressed GFP. Nuclei were revealed by staining with 4,6-diamidino-2-phenylindole (DAPI). Arrowheads indicate transfected cells.

Other mutations downstream of codon 1500 that occur less frequently than the 4666insA mutation also may differentially affect the wild-type *APC* and APC_{AS9}. The finding that 4616delAG also occurred on the APC_{AS9} allele supports this hypothesis. It is also supported by observations that somatic mutations of *APC* that are downstream of codon 1500 are rare in colorectal tumors (Nagase et al. 1992; Powell et al. 1992; Miyaki et al. 1994). Moreover, patients carrying germline *APC* mutations downstream of codon 1500, including those in a family reported to carry the 4666insA mutation, usually develop AFAP (Friedl et al. 1996; van der Luijt et al. 1996; Walon et al. 1997; Soravia et al. 1998). Future studies of derivatives of human colon cancer cell lines that can be induced to express different forms of APC could further clarify the tumor-suppression function of these APC variants.

These findings provide a model for AFAP in patients carrying APC_{AS9}. We propose that these patients develop fewer tumors, and at older ages, than patients with typical FAP because, although patients with AFAP already have a germline *APC* mutation, somatic mutation of both *APC* alleles is necessary prior to tumorigenesis. Nevertheless, patients with AFAP are still predisposed to colorectal tumorigenesis, because APC_{AS9} is more easily inactivated than is wild-type *APC,* by mutations that do not significantly affect the tumor-suppressor function of the wild-type *APC* (fig. 4). This model for AFAP

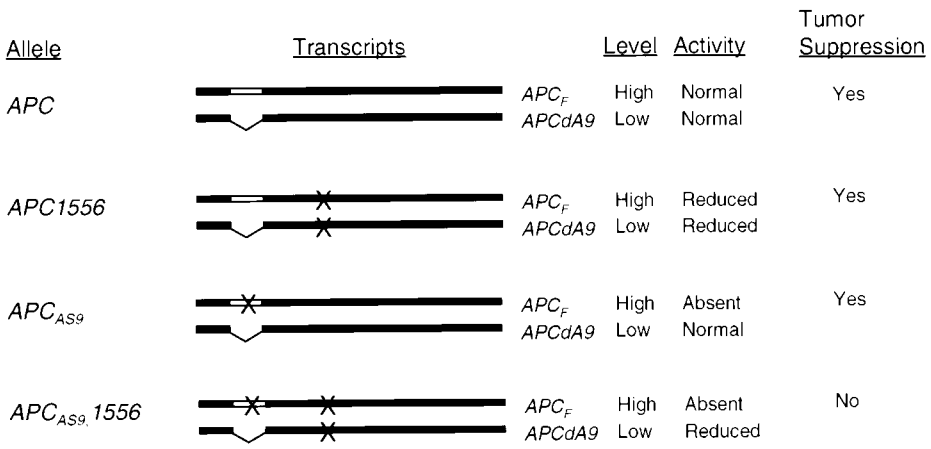

Figure 4 Inactivation of APC_{AS9}, but not wild-type *APC*, by the 4666insA mutation. "APC_F" indicates the full-length *APC* mRNA. " × " indicates mutations. "Tumor Suppression" indicates whether each *APC* allele can function as a tumor suppressor, which is determined by the combined activities of transcripts of each allele. The mutation 4666insA slightly reduces the activity of proteins encoded by both APC_F and *APCdA9* and does not abolish the tumor-suppressor function of *APC*. APC_{AS9} does not express full-length APC protein, because its APC_F expresses a severely truncated protein owing to the mutation in the alternatively spliced region of exon 9. However, APC_{AS9} expresses APCdA9 identical to that expressed by the wild-type *APC* and has reduced but sufficient tumor-suppressor activity. Further decrease of the reduced tumor-suppressor activity of APC_{AS9} by the 4666insA mutation causes APC_{AS9} to have insufficient tumor-suppressor activity.

caused by APC_{AS9} also may explain AFAP caused by $APC_{1–4}$. Alternative splicing of the first four coding exons of *APC* has been demonstrated, and the 4666insA somatic mutation has been found in the $APC_{1–4}$ allele in tumors (Samowitz et al. 1995; Spirio et al. 1998). However, our model does not readily explain AFAP caused by APC_{3H}. Although somatic inactivation of APC_{3H} is probably required for tumor formation, mutation such as 4666insA is unlikely to inactivate APC_{3H}. Most reported APC_{3H} alleles have germline mutations downstream of codon 1556. Therefore, a somatic 4666insA mutation in these alleles will result in *APC1556*, which still has tumor-suppressor activity. Thus, the data support a model requiring somatic inactivation of both *APC* alleles, for tumor formation in patients with AFAP, although the mechanism for the increased somatic inactivation of alleles with germline mutation in different regions of *APC* may be different.

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Appendix

Summary of *APC* Alleles, mRNA, and cDNA Described in this Paper

$APC_{1–4}$	*APC* alleles that carry germline mutations in the first four coding exons
APC_{AS9}	*APC* alleles that carry germline mutations in the alternatively spliced region of exon 9
APC_{3H}	*APC* alleles that carry germline mutations in the 3′ half of the coding region
APC_{AFAP}	*APC* alleles that usually cause AFAP, including $APC_{1–4}$, APC_{AS9}, and APC_{3H}
APC1556	*APC* allele or cDNA that carries the 4666insA mutation
APC_{AS9},*1556*	*APC* alleles that carry germline mutations in the alternatively spliced region of exon 9 and the 4666insA mutation
APCdA9	*APC* mRNA or cDNA that does not have the alternatively spliced region of exon 9
APCdA,1556	*APC* mRNA or cDNA that does not have the alternatively spliced region of exon 9 and carries the 4666insA mutation

Electronic-Database Information

The accession number and URL for data in this article is as follows:

Online Mendelian Inheritance in Man (OMIM), http://www3.ncbi.nlm.nih.gov/Omim/ (for *APC*/FAP [MIM 175100])

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