MOLECULAR DIAGNOSIS OF FAMILIAL ADENOMATOUS POLYPOSIS

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Abstract *Background.* Familial adenomatous polyposis is an inherited disease characterized by multiple colorectal tumors. The diagnosis has classically been based on the detection of multiple colorectal adenomas. The recent identification of germline mutations of the *APC* gene in patients with familial adenomatous polyposis makes presymptomatic molecular diagnosis possible, but the widespread distribution of the many mutations within this very large gene have heretofore made the search for such mutations impractical. We describe a novel approach that allows molecular genetic diagnosis in the majority of patients with the disease.

Methods. We screened 62 unrelated patients from the Johns Hopkins Familial Adenomatous Polyposis Registry for germline *APC* mutations. Primary screening was accomplished by analysis of protein synthesized in vitro from surrogate *APC* genes. In addition, the relative amount of transcript from each APC allele was determined with an allele-specific–expression assay.

Results. The protein assay revealed truncated protein in 51 of the 62 patients (82 percent). In 3 of the 11 remaining patients, the allele-specific–expression assay revealed significantly reduced expression of one allele of the *APC* gene. The use of these two assays in combination successfully identified germline *APC* mutations in 87 percent of the 62 patients.

Conclusions. The protein and allele-specific–expression assays provide a practical and sensitive method for molecular diagnosis of familial adenomatous polyposis. This approach will facilitate care, allowing routine testing of subjects at risk and genetic confirmation of spontaneous mutations. (N Engl J Med 1993;329:1982-7.)

FAMILIAL ADENOMATOUS POLYPOSIS is a dominantly inherited syndrome characterized by the progressive development of hundreds of adenomatous colorectal polyps, some of which inevitably progress to cancer. Although the clinical manifestations of this syndrome and its variants (e.g., Gardner's syndrome and Turcot syndrome) have been known for many years,[1] the diagnosis still relies largely on the detection of numerous colorectal polyps during the second or third decade of life. Today, with familial adenomatous polyposis affecting nearly 1 in 8000 people[2] and twice that many at risk, there are more than 50,000 people in the United States alone whose families could benefit from genetic testing.

The first step toward genetic testing was achieved when the inheritance of familial adenomatous polyposis was linked to a small region of chromosome 5 (5q21).[3-5] This observation set the groundwork for linkage studies with nearby polymorphic DNA markers. Although linkage analysis is useful in some situations, it can benefit only a minority of kindreds with familial adenomatous polyposis.[6-8] Direct genetic testing became feasible when the *APC* gene on chromosome 5q21 was found to be mutated in the germline of patients with the syndrome.[9-12] The *APC* gene is also mutated frequently and early during sporadic colorectal tumorigenesis.[12-14]

Analyses of the entire coding region of the *APC* gene have detected mutations in 30 to 60 percent of patients with familial adenomatous polyposis, depending on the screening method used.[15-17] These analyses were complicated by the varied nature of the mutations, which were distributed over a large portion of the *APC* gene, which encompasses more than 8500 base pairs (bp) of open reading frame. Moreover, these mutations were mostly changes in single base pairs, small insertions, or small deletions.

Consequently, we sought to devise a rapid and sensitive method for the detection of *APC* gene mutations. Because the majority of mutations in patients with familial adenomatous polyposis result in truncations of the *APC* gene products due to frameshifts, nonsense mutations, and splice-site changes, we developed a method based on the examination of APC proteins synthesized in vitro and endogenous APC transcripts. The usefulness of this approach for the genetic diagnosis of familial adenomatous polyposis was then evaluated in 62 patients.

METHODS

Study Group

We studied the most recent 62 unrelated patients (37 female and 25 male patients) enrolled in the Johns Hopkins Familial Adenomatous Polyposis Registry from whom blood samples could be obtained. The patients' mean age was 41 years (range, 16 to 67). Fourteen patients had spontaneous mutations. All 62 patients were confirmed to have classic adenomatous polyposis as defined by the presence of more than 100 colorectal polyps at the time of endoscopy, radiologic study, or examination of resected colons. The adenomatous nature of the polyps was documented by histopathological analysis.

We also studied nine unrelated normal subjects (one woman and eight men; mean age, 32 years; range, 20 to 43) and seven relatives (five women and two men; mean age, 56 years; range, 32 to 75) of the three patients with familial adenomatous polyposis in whom an alteration was detected on the allele-specific–expression assay. Affected relatives met the criteria for classic adenomatous polyposis described above. Informed consent in accord with institutional policy was obtained from each subject before collection of the blood samples.

Preparation of Templates

Blood samples from 45 of the 62 patients with familial adenomatous polyposis were collected in EDTA and stored overnight at ambient temperature. Genomic DNA was prepared by Chelex ex-

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traction of 30 μl of whole blood as described previously.[18] RNA was isolated by the acid guanidium isothiocyanate–phenol–chloroform extraction method[19] from peripheral-blood mononuclear cells prepared from 10 ml of whole blood by Ficoll–Hypaque (Histopaque-1077, Sigma Chemical, St. Louis) gradient centrifugation. Blood samples from the normal subjects and the patients' relatives were processed in a similar manner.

For the remaining 17 patients, lymphoblastoid cell lines were established by Epstein–Barr virus–induced immortalization of lymphocytes isolated from peripheral blood. RNA and DNA were extracted from these cells as described.[19,20]

In Vitro Synthesized-Protein Assay

For the purposes of analysis with the polymerase chain reaction (PCR), the *APC* gene was divided into five overlapping segments containing codons 1 to 804, 686 to 1217, 1099 to 1693, 1555 to 2256, and 2131 to 2843. The primers used for PCR amplification were

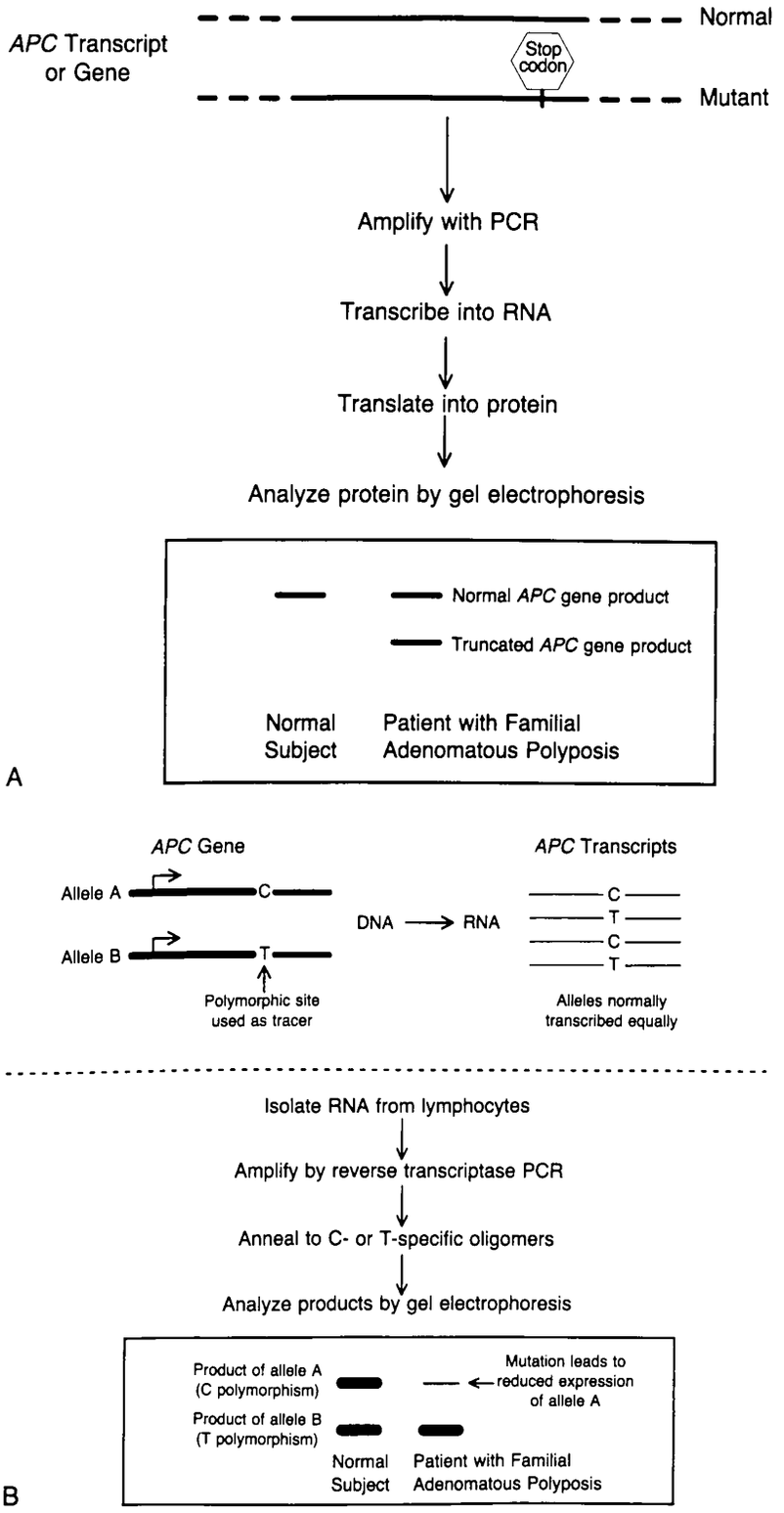

Figure 1. Principles of the In Vitro Synthesized-Protein Assay (Panel A) and the Allele-Specific–Expression Assay (Panel B).

In Panel A, for the protein assay, the *APC* gene is divided into five overlapping segments encompassing the entire coding region of the gene. These regions are amplified with specially designed PCR primers that place the necessary transcriptional and translational regulatory sequences at the 5′ end of the PCR product. Radiolabeled protein is synthesized in vitro from these surrogate genes in a simple one-step coupled transcription–translation reaction (illustrated as two steps). Truncating mutations can then be identified as smaller protein products after gel electrophoresis and autoradiography. The stop codon represents a typical truncating *APC* mutation — for example, a change in a single base pair that creates a premature translation-termination codon. In Panel B, for the allele-specific–expression assay, every normal cell has two copies of the *APC* gene that are identical except for occasional polymorphisms of a single base pair (cytosine [C] or thymidine [T] in this example). Normally, both alleles of the *APC* gene are equally represented in the RNA fraction of the cell. However, some cases of familial adenomatous polyposis are caused by mutations that lead to reduced levels of normal *APC* transcript from one allele. This results in an imbalance in the representation of the transcripts from the two alleles. This altered allele ratio in RNA can be detected with the allele-specific–expression assay (outlined below the dotted line). First, RNA is isolated from peripheral-blood mononuclear cells. *APC* transcripts are converted to complementary DNA and amplified by reverse transcriptase PCR. The PCR products are then annealed with a common 9-bp oligomer and two different-sized allele-specific oligomers (8 and 10 bp). After ligation, these oligomers will yield 17-bp and 19-bp products corresponding to alleles A and B that can be distinguished by gel electrophoresis. The box shows the expected results from a normal subject and from a patient with familial adenomatous polyposis who has a mutation that leads to the reduced expression of the normal transcripts of allele A.

designed to introduce a T7 promoter sequence for the initiation of transcription by T7 RNA polymerase, as well as a consensus sequence for the initiation of translation.[21] Segment 1 was isolated from complementary DNA templates prepared by reverse transcription of messenger RNA (mRNA). Segments 2 to 5 were isolated directly from genomic DNA.

PCR products were used directly (without purification) as templates in coupled transcription–translation reactions (Promega, Madison, Wis.) with 40 μCi of ^{35}S-labeled methionine (ICN, Irvine, Calif.) and incubated for one hour at 30° C. The samples were diluted in sample buffer, boiled for five minutes, and analyzed by electrophoresis on a sodium dodecyl sulfate–polyacrylamide gel with a gradient of 10 to 20 percent. The proteins were visualized by fluorography after the gel had been impregnated with ENHANCE (New England Nuclear, Boston).

Allele-Specific–Expression Assay

The two-site polymorphisms used in this assay were silent changes of a single base pair, one in exon 11 (codon 486) and one in exon 13 (codon 545).[13] Informative heterozygous cases were first sought by analyzing amplified *APC* exons 11 and 13 from genomic DNA. The genomic PCR product was added to a modified allele-specific ligation assay[22] (and Jen J, et al.: unpublished data). A common 9-bp ^{32}P-labeled oligomer and two different-sized allele-specific oligomers (8 and 10 bp) were used in the ligation assay. Ligation products were separated by polyacrylamide–urea (8 M) sequencing gels, and the abundance of each allele was determined by the relative amount of allele-specific ligation product (19 bp for allele A and 17 bp for allele B). Quantitation was achieved with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). The PCR products of segment 1 (derived from mRNA, as for the protein assay) were then analyzed in the same quantitative ligation reaction to determine the relative abundance of *APC* transcripts expressed from each allele. The sequences of the PCR primers used in this study as well as a detailed protocol for PCR and the analysis of the PCR products are available from the National Auxiliary Publications Service.*

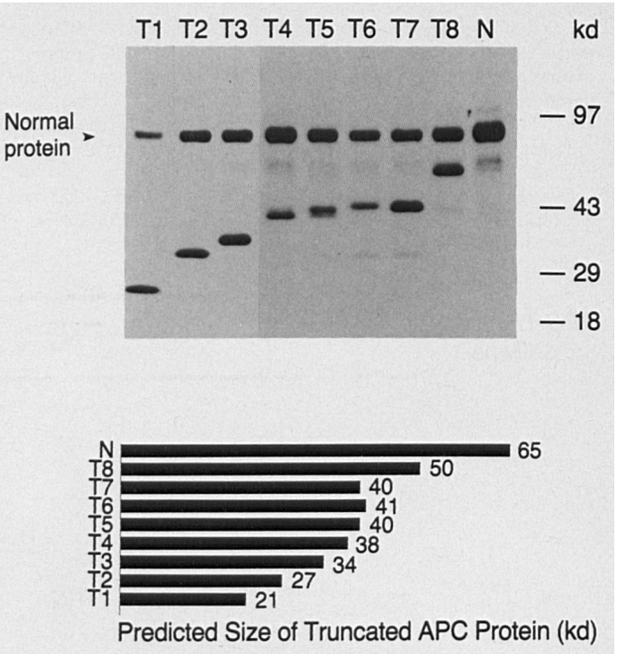

Figure 2. Protein Assay for the Detection of Known Truncating *APC* Mutations.

Representative samples of sporadic colorectal tumors (T1 through T8), known to have truncating mutations from sequence analysis, demonstrate the expected truncated APC proteins in segment 3. A substantial amount of normal, full-length APC protein is noted in the remaining normal alleles. A sample of normal tissue (N) is also shown. The numbers to the right of the horizontal bars indicate the predicted size of the truncated APC protein.

Results

The observation that mutations of the *APC* gene in patients with familial adenomatous polyposis almost always result in a truncated protein prompted us to develop an assay based on an examination of the *APC* gene product. This assay was made possible by two advances in molecular genetics. First, PCR allows the rapid isolation of specific gene segments from samples obtained from patients.[23] Second, the isolated gene segments can be used to produce proteins with in vitro transcription and translation reactions.[24] Truncating mutations can then easily be identified as small protein products after gel electrophoresis and autoradiography. A diagram of this procedure is shown in Figure 1A.

To validate the in vitro synthesized protein assay, we first analyzed 20 sporadically occurring colorectal tumors that were shown by sequence analysis to have truncating *APC* mutations.[13] In each tumor, a specific truncated protein corresponding to the predicted size of the mutant product was identified (examples are shown in Fig. 2). A full-length protein product of the remaining normal allele was also found in each tumor.

We next evaluated the efficacy of this approach for the molecular diagnosis of familial adenomatous polyposis from peripheral-blood samples. Analysis of the entire coding region of the *APC* gene with the protein assay identified truncating mutations in 51 patients (82 percent) (examples are shown in Fig. 3). Twelve of these mutations were from patients with spontaneous mutations (i.e., patients with first-generation mutations). The 51 mutations were distributed over the first four segments, with 29, 10, 11, and 1 in segments 1, 2, 3, and 4, respectively.

Since some patients with familial adenomatous polyposis may have promoter or splicing mutations that lead to reduced levels of normal *APC* transcripts, we also addressed ways to identify such mutations. Because such mutations, which influence the expression of a single allele, could result at most in a 50 percent decrease in the total amount of transcript, we devised an assay for the individual expression of each of the two alleles. We took advantage of polymorphisms affecting a single base pair to create an allele-specific–expression assay to detect this type of alteration (Fig. 1B). The accuracy of this assay was demonstrated in an allele-mixing experiment. RNA from two patients, each homozygous for different alleles at the polymorphic site in exon 11, was mixed together in defined

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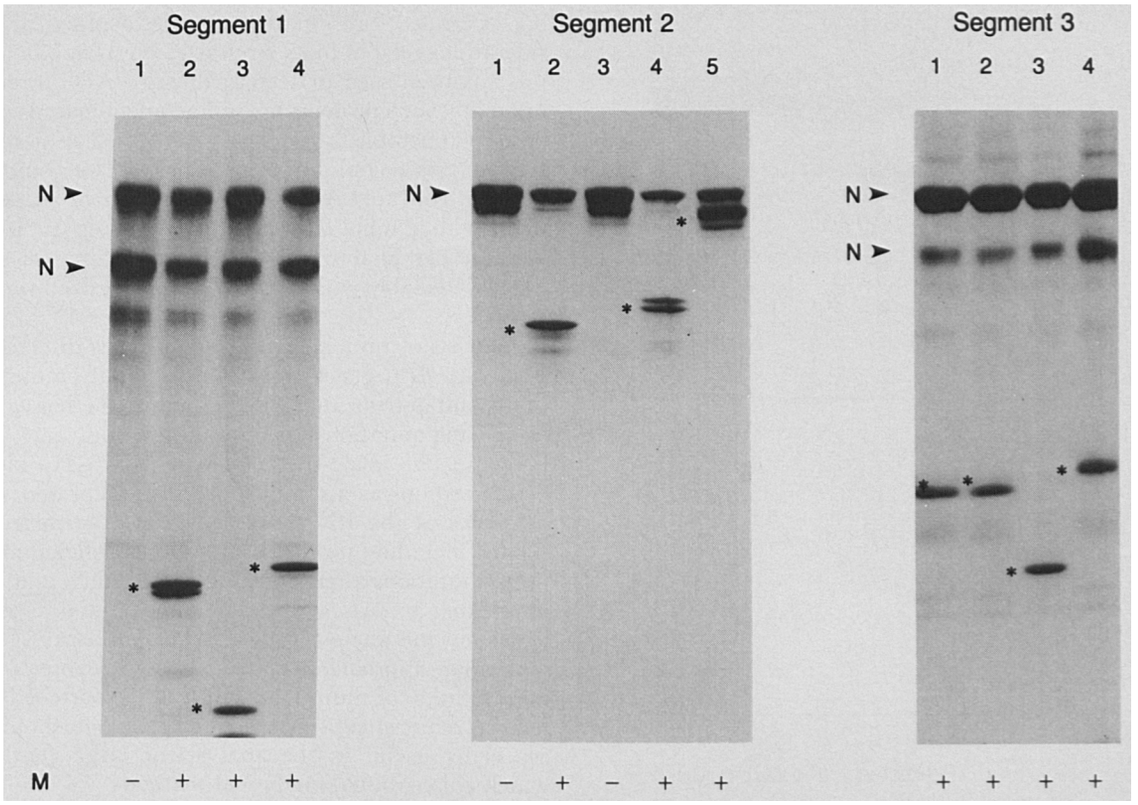

Figure 3. Protein Assay for the Detection of *APC* Mutations in Patients with Familial Adenomatous Polyposis.

Truncated APC proteins in segments 1, 2, and 3 were detected in representative patients with familial adenomatous polyposis. Novel bands corresponding to the truncated proteins are indicated by asterisks. The normal, full-length APC protein from the remaining unaltered *APC* allele is noted, as are background bands (also labeled N) that were present in all lanes. The background bands probably result from the internal initiation of protein translation. The presence or absence of a detectable mutation (M) in a given assay (lane) is indicated on the bottom by a plus or a minus sign.

ratios, amplified, and analyzed by the allele-specific–expression assay. The relative amounts of each allele determined by the assay were similar to the predicted ratios ($r = 0.997$) (Fig. 4).

We then used the assay to evaluate the 11 patients with familial adenomatous polyposis who had no detectable abnormalities in APC protein, as well as normal subjects. Seven patients and six normal subjects were heterozygous for at least one of the two polymorphisms. The relative allele ratio of the genomic DNA from these 13 subjects and of the RNA from the 6 normal subjects was 1.0 ± 0.2 (mean $\pm$SD). Three of the patients with familial adenomatous polyposis had a significant reduction in the expression of one allele (Fig. 5). In each of these three patients, the ratio of allele abundance in the expressed RNA template was significantly different from that of the normal subjects and from that found with the use of genomic DNA instead of RNA as a template ($P<0.001$ by two-tailed unpaired Student's t-test). At least one other affected family member from the kindreds of these three patients was also studied and found to have a similar reduction in the expression of the same allele, demonstrating the expected inheritance. Used in combination, the protein assay and the allele-specific–expression assay successfully identified *APC* mutations in 87 percent of the 62 kindreds with familial adenomatous polyposis tested.

Discussion

We have described practical molecular genetic methods for identifying inactivating mutations in large genes that have several advantages over currently available genetic methods. Although useful, genetic-linkage analysis cannot be applied when kindreds are small, the requisite number of affected family members is not available, or polymorphic markers are uninformative. Furthermore, linkage analysis cannot be applied when a spontaneous mutation is suspected. Indeed, spontaneous mutations account for approximately one third of the cases of familial adenomatous polyposis,[25] and they were present in 14 of the patients in this study, 12 of whom had identifiable *APC* mutations. In addition, because linkage analysis is indirect, some degree of uncertainty always remains. Several studies have described the direct detection of *APC* mutations in patients with familial adenomatous polyposis, with detection rates ranging from 10 to 60 percent, depending on the method used[9,15-17,26-29]; the methods were generally very labor intensive and could miss a substantial fraction of subtle changes in single base pairs. In many patients the entire *APC* gene was

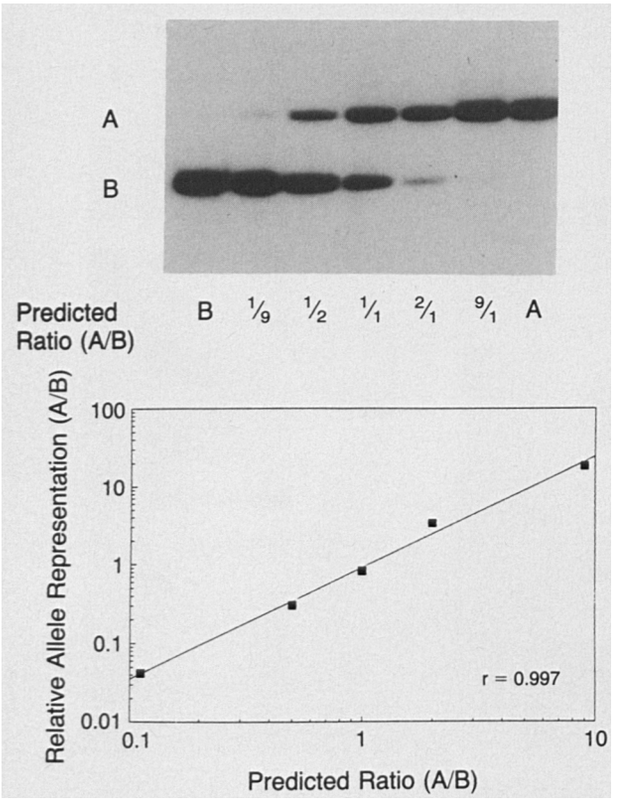

Figure 4. Allele-Mixing Analysis to Determine the Accuracy of the Allele-Specific–Expression Assay.

Defined amounts of RNA from two patients, each homozygous for one allele (A or B) at the polymorphic site in exon 11, were used as templates for amplification and subsequent ligation reactions. The predicted ratio is based on the relative amount of each allele added to the assay. A linear correlation between the predicted ratio and the assay result is apparent.

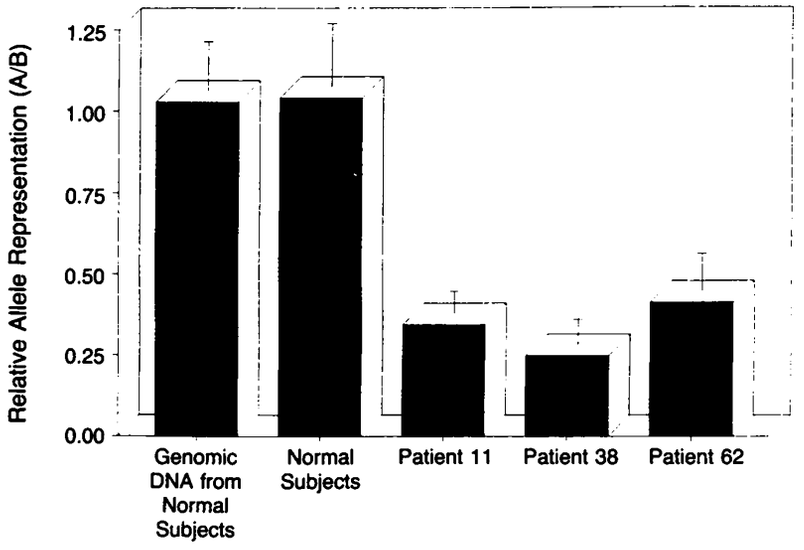

Figure 5. Detection of Altered *APC* Transcripts by the Allele-Specific–Expression Assay.

Reduced expression of an *APC* allele was detected in three patients with familial adenomatous polyposis by allele-specific–expression analysis with the exon 11 polymorphism. The average allele ratio is shown for 28 samples of genomic DNA from 21 normal subjects and 11 samples of RNA from 4 normal subjects. The ratio for each patient was derived from four assays. Results are given as means ±SD.

not examined, presumably because of practical considerations related to its large size. Western blot analysis has been used to detect truncated APC proteins, but many such proteins in familial adenomatous polyposis are unstable in vivo, precluding their demonstration by this technique.[30] For example, we could not identify truncated APC proteins in three of seven patients with familial adenomatous polyposis studied by Western blot analysis, but mutations in all seven were easily detectable with the in vitro synthesized-protein assay.

Analysis of proteins whose synthesis is directed by surrogate *APC* genes can rapidly identify mutations that result in truncated APC proteins, whether caused by splicing mutations, point mutations, or frameshifts. Likewise, the allele-specific–expression assay identified mutations as an imbalance in the representation of alleles at the RNA-transcript level. A variety of events, including promoter mutations, splicing mutations, mutations altering transcript stability, and even imprinting abnormalities, have the potential to be detected by the allele-specific–expression assay. These molecular approaches should be applicable to the identification of mutations that lead to shortened proteins or reduced expression in any gene, but should be especially useful in the analysis of large genes for which conventional analytical methods are too labor intensive. The mutations in the recently isolated genes responsible for neurofibromatosis type 2[31,32] and von Hippel–Lindau disease[33] are predominantly of the type that would be detected by our assays.

The detection of *APC* gene alterations in 87 percent of patients with familial adenomatous polyposis illustrates the usefulness of this approach as a genetic test for the disease. Moreover, the ability to use the assay for prenatal diagnosis could be important to patients planning a family. In providing a test for presymptomatic testing, our assays should have practical benefits for kindreds with familial adenomatous polyposis. Registry records for the 54 patients in whom we detected *APC* gene abnormalities show that there are at least 280 relatives at risk for the disease who can now be tested. The 166 subjects who are under the age of 20 years should gain the most from this analysis. Subjects who have a positive test can at least be spared the anxiety associated with not knowing whether they have the disease. They can also benefit from improved management, because early diagnosis should ensure that appropriate preventive measures are taken well before the development of colorectal cancers. Finally, the importance of preclinical testing is heightened by recent studies show-

ing promise in the pharmacologic treatment of polyposis.[34-38] Such treatment of subjects who have inherited a mutated *APC* gene should be more efficacious if initiated before polyps appear.

We are indebted to the patients, their families, and their physicians and to the Delaware Registry for Hereditary Colorectal Cancer for providing blood samples and kindred information through the Johns Hopkins Familial Adenomatous Polyposis Registry.

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