

## DETECTION OF APC MUTATIONS IN FECAL DNA FROM PATIENTS WITH COLORECTAL TUMORS

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**ABSTRACT**

**Background** Noninvasive methods for detecting colorectal tumors have the potential to reduce morbidity and mortality from this disease. The mutations in the adenomatous polyposis coli (*APC*) gene that initiate colorectal tumors theoretically provide an optimal marker for detecting colorectal tumors. The purpose of our study was to determine the feasibility of detecting *APC* mutations in fecal DNA with the use of newly developed methods.

**Methods** We purified DNA from routinely collected stool samples and screened for *APC* mutations with the use of a novel approach called digital protein truncation. Many different mutations could potentially be identified in a sensitive and specific manner with this technique.

**Results** Stool samples from 28 patients with non-metastatic colorectal cancers, 18 patients with adenomas that were at least 1 cm in diameter, and 28 control patients without neoplastic disease were studied. *APC* mutations were identified in 26 of the 46 patients with neoplasia (57 percent; 95 percent confidence interval, 41 to 71 percent) and in none of the 28 control patients (0 percent; 95 percent confidence interval, 0 to 12 percent;  $P < 0.001$ ). In the patients with positive tests, mutant *APC* genes made up 0.4 to 14.1 percent of all *APC* genes in the stool.

**Conclusions** *APC* mutations can be detected in fecal DNA from patients with relatively early colorectal tumors. This feasibility study suggests a new approach for the early detection of colorectal neoplasms. (N Engl J Med 2002;346:311-20.)

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SEVERAL strategies for the early detection of colorectal tumors have been devised. Colonoscopy, sigmoidoscopy, and barium enemas are highly specific and sensitive tests for neoplasia,<sup>1-4</sup> but they are invasive and limited by the availability of experts in the procedures and patient compliance.<sup>5,6</sup> Testing for occult blood in the stool has been shown in some studies to reduce the incidence of and morbidity and mortality from colorectal cancer.<sup>7-11</sup> These fecal occult-blood tests are non-invasive and extremely useful but not sufficiently sensitive or specific for neoplasia.<sup>12-15</sup> Furthermore, some fecal occult-blood tests require patients to change their diet before testing or require multiple tests, potentially reducing compliance.<sup>5,16,17</sup> There is

thus a need to develop new screening tests that overcome these obstacles.

One of the most promising classes of new diagnostic markers consists of mutations in oncogenes and tumor-suppressor genes.<sup>18</sup> Because these mutations are directly responsible for neoplastic growth, they have clear advantages over indirect markers such as fecal occult blood. Several groups have reported that mutations in cancer-related genes can be detected in the stool of patients with colorectal cancer.<sup>19-33</sup> However, the sensitivities and specificities of these approaches have been limited by technical impediments or the low frequencies of detectable mutations in any specific gene.

The intent of our study was to develop a test based on a single gene that would facilitate the detection of colorectal tumors at an early stage of disease. The optimal gene for such studies is the adenomatous polyposis coli (*APC*) gene,<sup>34,35</sup> since mutations in this gene generally initiate colorectal neoplasia.<sup>36</sup> Other mutations are present only in the later stages of colorectal neoplasia, such as those in *p53*,<sup>37</sup> or may be present in non-neoplastic, hyperproliferative cells, such as those in *c-Ki-ras*.<sup>38-40</sup> However, the detection of mutations in *APC* presents extraordinarily difficult technical challenges. Unlike mutations in *c-Ki-ras*, which have been used for most previous studies because mutations are clustered at two codons, mutations in *APC* can occur virtually anywhere within the first 1600 codons of the gene.<sup>41</sup> Moreover, the type of mutation (base substitutions or insertions or deletions of diverse length) varies widely among tumors. Although such *APC* mutations can be detected relatively easily in tumors, where they are present in every neoplastic cell, they are much harder to detect in fecal DNA,

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**DETECTION OF APC MUTATIONS IN FECAL DNA FROM PATIENTS WITH COLORECTAL TUMORS**

**TABLE 1. CHARACTERISTICS OF 46 PATIENTS WITH NEOPLASIA.\***

PATIENT No.	SEX/ AGE (YR)	SITE	DUKES' STAGE OR HISTOLOGIC FINDINGS	DIAMETER OF LESION	MUTANT APC GENES		MUTATION IDENTIFIED
					APC GENE	%	
1	F/74	Sigmoid colon	Tubulovillous adenoma	3.0	1.4	NF	Not applicable
2	M/56	Cecum	Tubular adenoma	1.0	0.6	1.2	GGA→TGA at position 1450
3	M/57	Splenic flexure	B2 (T3N0M0)	6.7	6.4	NF	Not applicable
4	F/47	Rectum	B2 (T3N0M0)	NR	75.6	1.3	ATA→TA at position 1286
5	F/67	Rectum	B2 (T3N0M0)	1.1	2.5	14.1	CAA→TAA at position 1303
6	F/70	Ascending colon	Tubular adenoma	1.0	1.3	1.9	GAG→G at position 1463
7	M/64	Rectum	B2 (T3N0M0)	1.3	800.0	0.4	GAA→TAA at position 1309
8	F/52	Rectum	Villous adenoma	3.0	26.6	1.1	GAAAAAACT→GAAAAAACT at position 1554
9	M/76	Sigmoid colon	B2 (T3N0M0)	1.3	2.5	NF	Not applicable
10	F/46	Rectum	Tubular adenoma	2.5	5.0	0.5	TCA→TGA at position 1346
11	F/52	Sigmoid colon	B2 (T3N0M0)	3.5	21.9	5.6	GAA→TAA at position 1295
12	F/36	Rectum	B2 (T3N0M0)	1.3	40.6	NF	Not applicable
13	M/54	Descending colon	Tubular adenoma	2.0	1.6	1.0	TTA→TT at position 1489
14	M/50	Rectum	B2 (T3N0M0)	0.9	18.8	NF	Not applicable
15	M/45	Rectum	B2 (T3N0M0)	0.5	32.7	NF	Not applicable
16	F/61	Rectum	B2 (T3N0M0)	NR	0.1	NF	Not applicable
17	M/64	Rectum	B2 (T3N0M0)	1.7	133.4	3.1	GAA→TAA at position 1353
18	M/84	Ascending colon, transverse colon†	Tubular adenoma	1.0, 1.5†	0.1	NF	Not applicable
19	M/50	Rectum	B2 (T3N0M0)	3.7	0.1	6.6	AAAGAAAAGA→AAAGA at position 1309
20	M/59	Descending colon	Tubular adenoma	1.0	0.2	NF	Not applicable
21	F/68	Ascending colon	B2 (T3N0M0)	1.7	0.2	NF	Not applicable
22	M/64	Rectum	B2 (T3N0M0)	2.4	5.1	NF	Not applicable
23	M/69	Rectum	B2 (T3N0M0)	5.0	1.5	1.2	AAAGAAAAGA→AAAGA at position 1309
24	M/50	Rectum	B2 (T3N0M0)	1.6	57.4	NF	Not applicable
25	M/58	Sigmoid colon	Tubular adenoma	1.0	1.4	1.2	GAG→G at position 1463
26	M/63	Rectum	B2 (T3N0M0)	0.8	9.4	NF	Not applicable
27	F/61	Rectum	Villous adenoma	4.5	0.2	NF	Not applicable
28	F/52	Ascending colon	Tubulovillous adenoma	2.0	3.7	NF	Not applicable
29	F/52	Transverse colon	Tubular adenoma	4.5	591.4	0.5	CGA→TGA at position 1450
30	M/47	Rectum	B2 (T3N0M0)	3.0	9.7	3.6	CAG→TAG at position 1367, AGT→AG at position 1411‡
31	M/70	Sigmoid colon	B2 (T3N0M0)	1.7	9.0	0.5	CAG→TAG at position 1480
32	M/53	Rectum	B2 (T3N0M0)	1.4	18.7	12.8	CAG→TAG at position 1406
33	M/54	Rectum	B2 (T3N0M0)	2.4	757.8	NF	Not applicable
34	M/49	Descending colon, descending colon†	Tubular adenoma	1.0, 1.0†	1.2	NF	Not applicable
35	F/62	Rectum	Villous adenoma	3.0	1.2	NF	Not applicable
36	M/78	Hepatic flexure	Tubulovillous adenoma	2.5	5.8	NF	Not applicable
37	F/74	Ascending colon, cecum†	Tubular adenoma	1.0, 1.0†	0.2	5.7	CAG→TAG at position 1294
38	F/42	Rectum	B2 (T3N0M0)	0.2	309.8	1.3	TCC→TC at position 1319
39	M/72	Ascending colon, ascending colon†	Tubular adenoma	1.0, 0.8†	0.3	NF	Not applicable
40	M/76	Rectum	B2 (T3N0M0)	1.4	3.5	1.6	GAA→TAA at position 1408
41	F/73	Ascending colon	Tubulovillous adenoma	1.0	0.2	1.8	GGA→TGA at position 1412
42	M/67	Rectum	B2 (T3N0M0)	2.4	300.0	1.9	CTTGATAGTT→CTTGAGTT at position 1394
43	M/60	Rectum	B2 (T3N0M0)	4.3	0.4	2.0	GAA→TAA at position 1317
44	M/70	Rectum	B2 (T3N0M0)	1.6	1.5	5.8	GAAAAAACT→GAAAAAACT at position 1554
45	M/75	Rectum	B2 (T3N0M0)	NR	59.2	0.9	TCA→TAA at position 1315
46	F/62	Rectum	B2 (T3N0M0)	0.6	6.6	2.3	AGA→TGA at position 1435

\*NF denotes none found, and NR not recorded.

†Two adenomas were found on colonoscopy.

‡Two different mutations were identified by the digital-protein-truncation test and confirmed by sequencing. It is not unusual for a single tumor to harbor mutations of both parental alleles.<sup>18,41</sup>

from cloned DNA were analyzed on a SCE-9610 96-well capillary electrophoresis system (SpectruMedix, State College, Pa.). In 19 cases, DNA was prepared from archived tumors, and *APC* fragments of approximately 200 bp were amplified and subjected to manual sequence analysis with ThermoSequinox (Amersham Pharmacia Biotech).

### Statistical Analysis

All statistical analyses employed Fisher's exact test to compare proportions. All reported P values are two-sided.

## RESULTS

### Development of the Digital-Protein-Truncation Assay

In order to detect *APC* mutations in fecal DNA we had to surmount two major technical obstacles. The first involved purification of DNA templates that were large enough to allow us to perform PCR on a substantial region of the *APC* gene. About 83 percent of the *APC* mutations in sporadic tumors occur between codons 1210 and 1581, an expanse of 1113 nucleotides.<sup>41</sup> For our analysis, it was important to amplify this region within a single PCR product rather than in multiple overlapping PCR products. The DNA molecules to be assessed must therefore be considerably larger than 1100 nucleotides. However, stool contains numerous inhibitors of DNA polymerase, and long PCR products, such as those of 1100 bp, are particularly sensitive to such inhibitors. The method we developed captured *APC* genes on magnetic beads that were coated with oligonucleotides corresponding to the region between codons 1210 and 1581. This allowed amplification of DNA fragments of the required size and concentration from all 74 stool samples analyzed. Patients with colorectal cancer had a median of 4.3 copies of the *APC* gene per milligram of stool (Table 1), and patients without colorectal neoplasia had a median of 2.3 copies of the *APC* gene per milligram of stool (Table 2).

The second technical hurdle was identifying mutations within these PCR products. Virtually all *APC* mutations result in stop codons caused by nonsense substitutions or small, out-of-frame deletions or insertions.<sup>41</sup> *APC* mutations can therefore be identified through in vitro transcription and translation of suitably engineered PCR products.<sup>44,45</sup> This "in vitro synthesized protein," or "protein-truncation," test is the standard method for genetic diagnosis of familial adenomatous polyposis. However, it could not be used to evaluate fecal DNA samples, because of the preponderance of wild-type sequences in such samples. In particular, the sensitivity of the conventional method is limited to mutations that occur in more than 15 percent of template molecules, whereas mutant *APC* genes were expected to be present at much lower frequency in fecal DNA (Fig. 1). We therefore developed a mod-

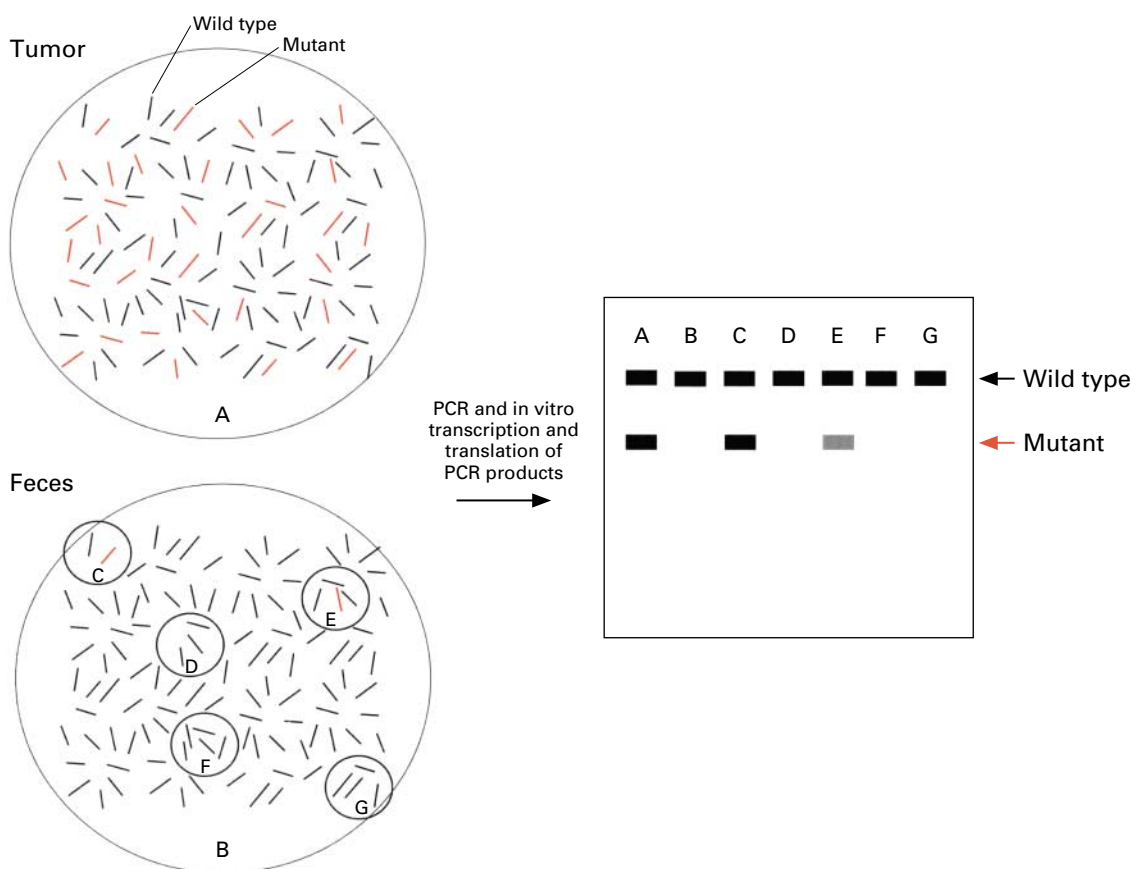
ification of the protein-truncation test, called digital protein truncation, which has considerably increased sensitivity (Fig. 1). In brief, a small number of template molecules were included in each reaction, and the protein products of each reaction were separated by polyacrylamide-gel electrophoresis. To increase the specificity of the digital-protein-truncation test and to control for polymerase-generated errors, we considered the test result to be positive for a mutation only when a truncated protein product of the same size was identified at least twice among the 144 reactions carried out on each sample.

### Analysis of Data from Patients with Cancer and Control Patients

Mutations were identified in 26 of the 46 stool samples from patients with neoplasia (57 percent;

TABLE 2. CHARACTERISTICS OF 28 CONTROL PATIENTS.

PATIENT NO.	SEX/AGE (YR)	REASON FOR COLONOSCOPY	APC GENE copies/mg of stool
47	M/26	Abdominal pain, rectal bleeding	40.1
48	F/27	Positive fecal occult-blood test	3.9
49	M/35	Rectal bleeding	6.1
50	F/36	Low abdominal pain	0.3
51	F/36	Family history of attenuated polyposis	2.3
52	F/41	Positive fecal occult-blood test	1.8
53	M/42	Family history of colorectal cancer	7.4
54	F/44	Rectal bleeding	2.3
55	F/44	Family history of colorectal cancer	17.4
56	F/47	Positive fecal occult-blood test	1.4
57	M/50	Family history of colorectal cancer	2.6
58	F/53	Family history of colorectal cancer	3.8
59	F/53	Family history of colorectal cancer	12.3
60	F/54	Family history of colorectal cancer	5.9
61	F/55	Family history of polyps	1.1
62	F/55	History of adenomas and nonadenomatous polyps	11.8
63	F/56	Family history of colorectal cancer	2.0
64	F/56	Low abdominal pain	1.0
65	F/58	Rectal bleeding	35.7
66	F/61	Positive fecal occult-blood test	0.7
67	F/62	Positive fecal occult-blood test	2.4
68	F/62	Family history of colorectal cancer	0.1
69	F/66	Rectal bleeding	1.0
70	F/69	Family history of colorectal cancer	0.6
71	M/69	Screening	0.3
72	F/70	Family history of colorectal cancer	0.5
73	F/72	Rectal bleeding	4.7
74	F/73	Low abdominal pain	0.4



**Figure 1.** The Digital-Protein-Truncation Test.

Digital protein truncation relies on the amplification of a small number of *APC* gene templates in each polymerase chain reaction (PCR), and the detection of truncated polypeptides generated by in vitro transcription and translation of the PCR products. The term "digital" is used to indicate that each well either contains or does not contain *APC* gene templates and that each protein-truncation test is therefore positive (1) or negative (0). The lines within each large circle represent single-stranded *APC* templates present in a population of DNA, with black and red lines indicating wild-type (normal) and mutant *APC* gene copies, respectively. In circle A, the mutant *APC* genes represent a large fraction of the total *APC* genes, as would be found in a tumor or in the blood cells of a patient with familial adenomatous polyposis. Analysis of the entire population of molecules with the use of PCR and in vitro transcription and translation readily reveals the mutant product, which is equivalent in intensity to the normal *APC* product (as shown in lane A of the schematic gel on the right). In circle B, the mutant *APC* genes represent only a small fraction of the total *APC* genes, as would be found in the feces of a patient with colorectal cancer. Analysis of the entire population of molecules with the use of PCR and in vitro transcription and translation does not reveal the mutant product, because it is present in too small a proportion of the molecules to create a detectable signal in the assay (as shown in lane B of the gel on the right). To reduce the complexity and thereby increase the ratio of mutant genes to normal genes, we sampled two to four molecules in each well, as indicated by the circles labeled C through G within circle B. Lanes D, F, and G represent wells with no mutant products; lane C represents a well in which one of the two *APC* templates was mutant; and lane E represents a well in which one of the four *APC* templates was mutant. The number of copies of the *APC* gene per well varies stochastically according to a Poisson distribution.

95 percent confidence interval, 41 to 71 percent) with use of the digital-protein-truncation assay. Representative positive results are shown in Figure 2. The average number of abnormal reactions in patients with positive results was 7.5 and ranged from 2 to 39 (of 144 total reactions carried out in each patient). No mutations were identified by the digital-protein-truncation assay in stools from the 28 control patients who did not have neoplastic disease (0 percent; 95 percent confidence interval, 0 to 12 percent;  $P < 0.001$ ). Positive results were obtained in 17 of the 28 patients with Dukes' stage B2 cancer (61 percent; 95 percent confidence interval, 41 to 79 percent) and 9 of the 18 patients who had adenomas that were at least 1 cm in diameter (50 percent; 95 percent confidence interval, 26 to 74 percent). In addition, 20 of 36 patients with neoplasms distal to the splenic flexure (56 percent; 95 percent confidence interval, 38 to 72 percent) had positive results, as did 6 of 10 patients with more proximal lesions (60 percent; 95 percent confidence interval, 26 to 88 percent). In the positive stool samples, 0.4 to 14.1 percent of all *APC* genes had mutations (Table 1).

#### Confirmation of Mutations

To confirm that the abnormal polypeptides detected by the digital-protein-truncation assay represented *APC* mutations, we determined the sequence of corresponding PCR products. In each of the 26 patients with positive tests, we found a mutation that was predicted to result in a truncated polypeptide of exactly the size found in the digital-protein-truncation assay (Fig. 3). The spectrum of mutations was broad (Fig. 4 and Table 1) and closely resembled those in sporadic colorectal neoplasms.<sup>41</sup>

We next sought to confirm that mutations identified in the stool were also present in the patients' tumors. Although in the majority of patients, tumor material suitable for mutational analyses was not available, we were able to evaluate *APC* mutations in the tumors of seven patients who had positive results on the digital-protein-truncation assay. The mutations in these tumors were identical to those found in the stool (Fig. 3). We also assessed the nature of *APC* mutations in tumors from 12 patients with negative results on the digital-protein-truncation assay. Tumors from 6 of these 12 patients had truncating mutations (at codons 1284, 1291, 1309, 1376, 1464, and 1488). Thus, 36 of the 46 patients with neoplasia (78 percent; 95 percent confidence interval, 65 to 89 percent) in our study were estimated to have mutations that could have been detected by the digital-protein-truncation assay (26 of the patients with positive test results plus 10 of the 20 patients with negative test results). This estimate of 78

percent is quite close to the value of 75 percent expected on the basis of previous studies.<sup>35,41</sup>

#### DISCUSSION

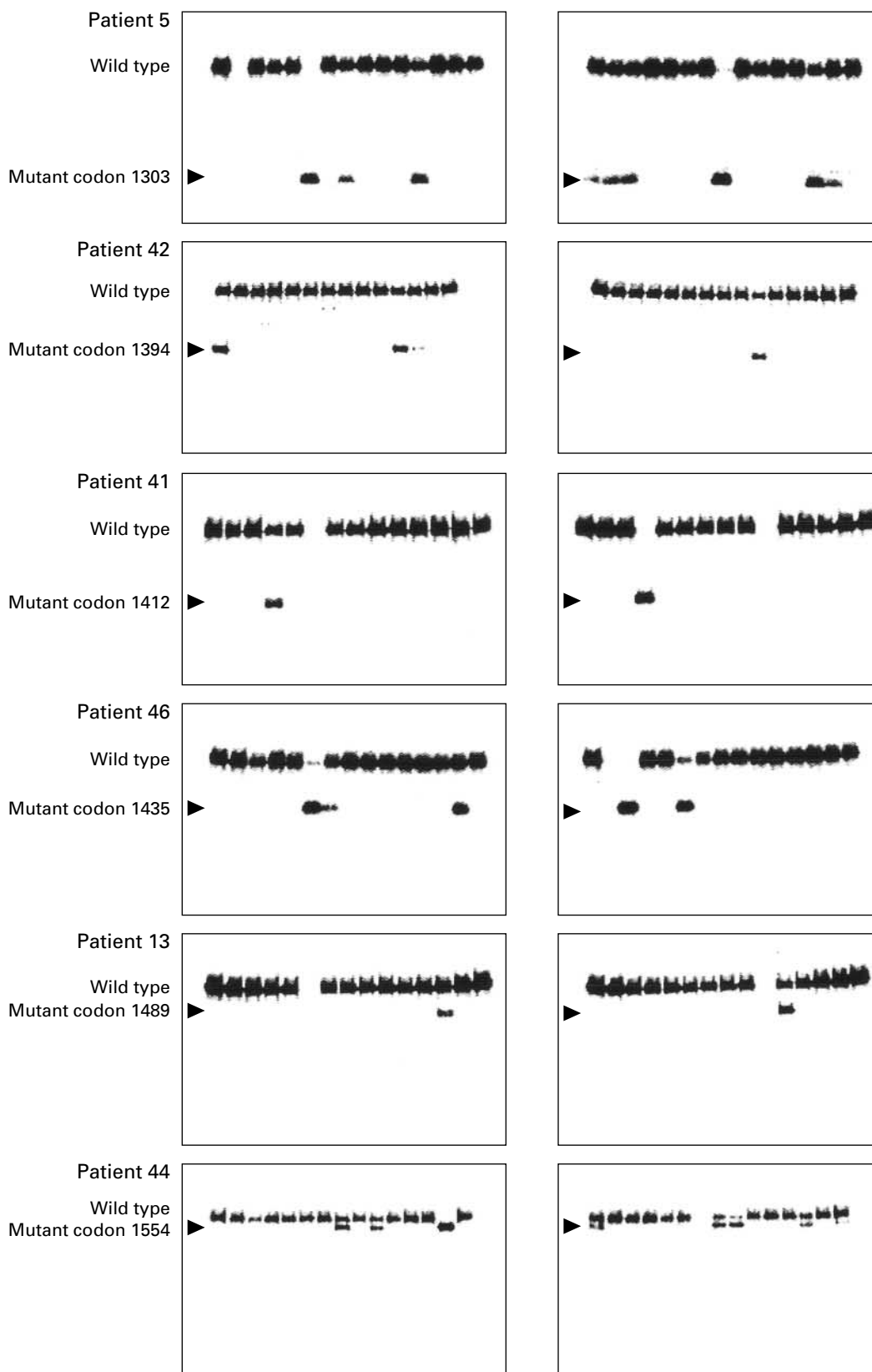
Our results show that PCR-amplifiable DNA fragments of more than 1100 bp could be purified from the stools of all patients studied, regardless of the presence or absence of a colorectal tumor or colonic adenoma. The fraction of mutant *APC* molecules in the samples from patients with neoplasia ranged from 0.4 to 14.1 percent. Knowledge gained from our study should be helpful in the design of future studies. For example, any technique to assess mutant DNA molecules in fecal DNA must have the capacity to distinguish 1 mutant molecule from more than 250 wild-type molecules if a sensitivity comparable to the one achieved in this study is to be achieved. By increasing the number of copies of *APC* examined, further increases in sensitivity should be achievable. Furthermore, our study focused on relatively early-stage lesions. Because of the high potential for cure by surgical or endoscopic removal of these lesions, their detection by noninvasive methods such as the digital-protein-truncation assay has the capacity to reduce morbidity and mortality in the future.

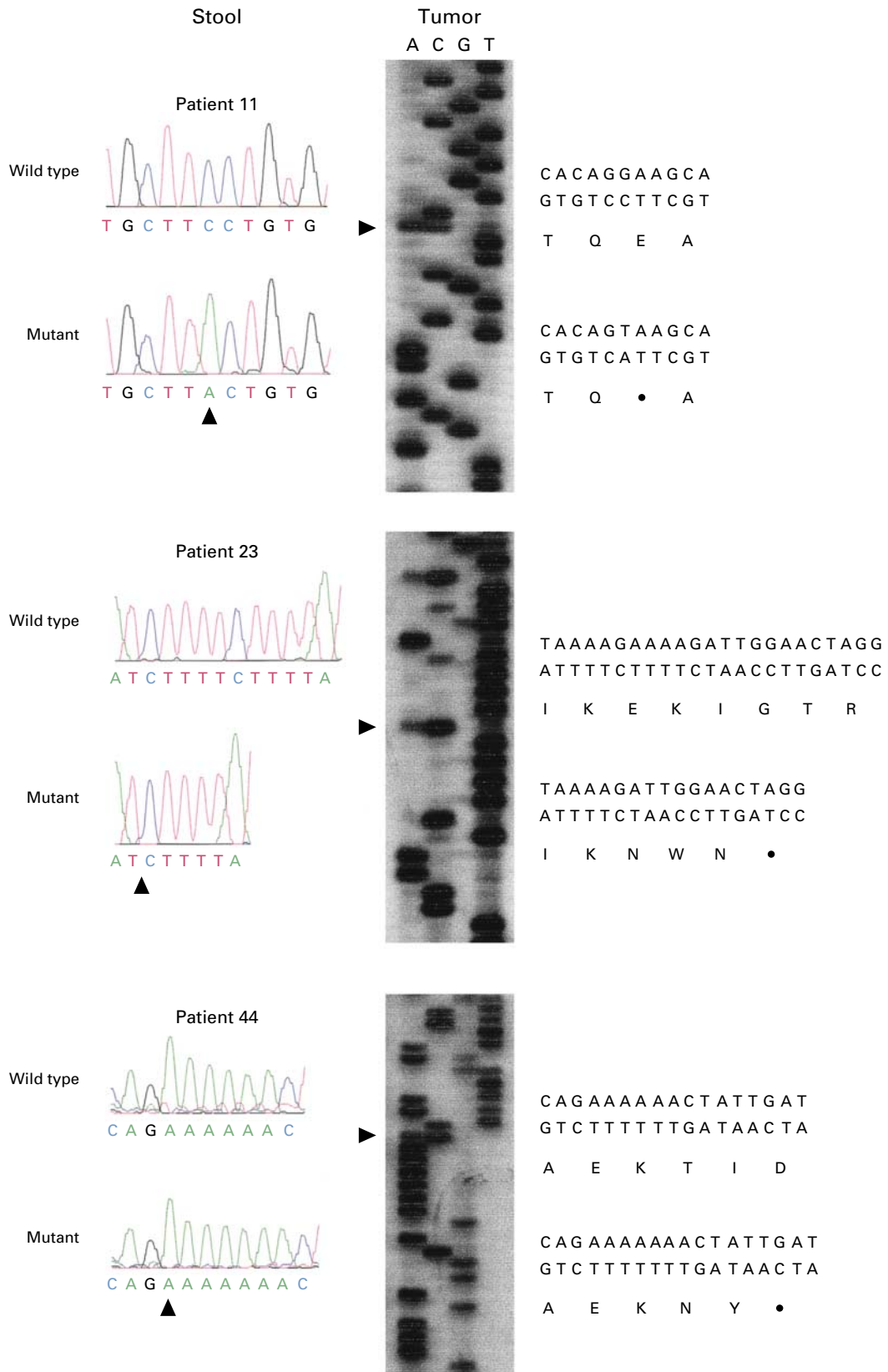
An important component of our study was the high specificity of the test: no *APC* alterations were identified in any of the 28 control samples from patients without neoplasia. Among the published studies of fecal-DNA mutations,<sup>19-33</sup> few used more than three stool samples from normal subjects as controls. In one such study, *c-Ki-ras* mutations were identified in 7 percent of the controls.<sup>30</sup> Nondysplastic aberrant crypt foci and small hyperplastic polyps, which occur relatively frequently in normal people but are not thought to be precursors of cancer, often contain *c-Ki-ras* mutations but not *APC* mutations,<sup>38-40</sup> a finding further emphasizing the value of *APC* for stool-based testing.

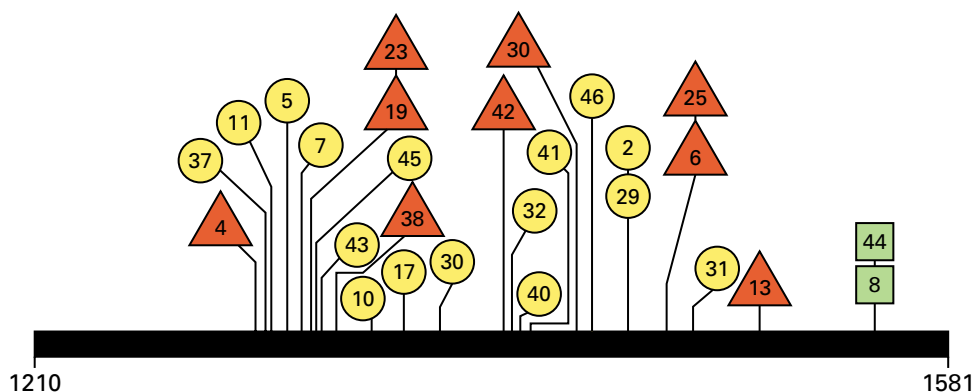
In summary, it is possible to detect *APC* mutations in fecal DNA in patients with potentially curable colorectal tumors. It is important to emphasize, however, that our study does not demonstrate that

**Figure 2 (facing page).** Examples of the Results of the Digital-Protein-Truncation Test in Six Patients with Truncating Mutations in *APC*.

The wild-type protein product is 43 kD. The products of in vitro transcription and translation from 30 individual reactions (15 reactions per panel) are shown for each patient, and the abnormal polypeptides are indicated by arrowheads. Because of the Poisson distribution of template molecules, an occasional lane contains no templates and is blank (e.g., lane 2 of the sample from Patient 5).







**Figure 4.** Spectrum of APC Mutations Identified between Codons 1210 and 1581 in Fecal DNA.

Twenty-seven different mutations were identified among the 26 patients with positive digital-protein-truncation tests. Mutations occurred in the form of deletions (red triangles), insertions (green squares), and base substitutions (yellow circles). The numbers within each symbol refer to the patient numbers shown in Table 1.

the digital-protein-truncation test is a clinically useful screening test. It was of interest that five of the control patients in our study underwent colonoscopy because of a positive fecal occult-blood test, whereas in another six, the reason for undergoing colonoscopy was rectal bleeding, which precludes fecal occult-blood testing. Although this result points to the potential value of a more specific genetically based test for screening feces, further studies will be required to determine whether the digital-protein-truncation test is as sensitive and specific as the fecal occult-blood test in persons at average risk. Because the digital-protein-truncation test is based on the

identification of abnormal proteins synthesized from mutant genes, the powerful new tools being developed for proteomics should be directly applicable to this approach in the future, further increasing its power.

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Drs. Kinzler and Vogelstein are entitled to royalties on sales of products related to the use of stool DNA for the diagnosis of cancer. Dr. Kinzler owns stock in and serves as a consultant to Genzyme and Exact Sciences. Dr. Vogelstein owns stock in and has served as a consultant to Genzyme and Exact Sciences. Dr. Schoetz owns stock in Exact Sciences, and the recruitment of patients and collection of samples at the Lahey Clinic were funded in part by Exact Sciences.

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**Figure 3 (facing page).** Mutations Producing Truncated Polypeptides in the Digital-Protein-Truncation Test.

Polymerase-chain-reaction (PCR) products that generated abnormal polypeptides in the digital-protein-truncation test were used for sequence analyses, as described in the Methods section. In each case, primers were chosen on the basis of the position of the mutation expected from the digital-protein-truncation results. For each patient, the upper chromatogram represents the wild-type sequence and the lower chromatogram depicts the mutant sequence (arrowheads indicate the site of the genetic alteration). Autoradiograms of sequencing gels from PCR products derived from tumor samples from the four patients are also shown; arrowheads indicate the mutations, which were identical to those observed in the stool samples. As expected, sequences of tumor-derived templates revealed the simultaneous presence of wild-type and mutant sequences. Examples of a base substitution (in the case of Patient 11), a 5-bp deletion (in the case of Patient 23), and an insertion of one base (in the case of Patient 44) are illustrated. All mutations resulted in stop codons (solid circles) immediately downstream from the mutations, as indicated on the right.

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