Somatic mutations of the *APC* gene in colorectal tumors: mutation cluster region in the *APC* gene

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ABSTRACT

We examined somatic mutations of the adenomatous polyposis coli (*APC*) gene in 63 colorectal tumors (16 adenomas and 47 carcinomas) developed in familial adenomatous polyposis (FAP) and non-FAP patients. In addition to loss of heterozygosity (LOH) at the APC locus in 30 tumors, 43 other somatic mutations were detected. Twenty-one of them were point mutations; 16 nonsense and two missense mutations, and three occurred in introns at the splicing site. Twenty-two tumors had frameshift mutations due to deletion or insertion; nineteen of them were deletions of one to 31 bp and three were a 1-bp insertion. One tumor had a 1-bp deletion in an intron near the splicing site. Hence, 41 (95%) of 43 mutations resulted in truncation of the APC protein. Over 60% of the somatic mutations in the *APC* gene were clustered within a small region of exon 15, designated as MCR (mutation cluster region), which accounted for less than 10% of the coding region. Combining these data and the results of LOH, more than 80% of tumors (14 adenomas and 39 carcinomas) had at least one mutation in the *APC* gene, of which more than 60% (9 adenomas and 23 carcinomas) had two mutations. These results strongly suggest that somatic mutations of the *APC* gene are associated with development of a great majority of colorectal tumors.

INTRODUCTION

We have recently isolated the adenomatous polyposis coli (*APC*) gene responsible for familial adenomatous polyposis (FAP), an autosomal dominant hereditary disease, characterized with hundreds to thousands of adenomatous polyps in colon and rectum (1, 2). Germ-line mutations in more than 90 unrelated FAP patients, the majority of which were considered to cause truncation of the predicted *APC* gene product, have been so far identified (3, Y. Nakamura, unpublished data). Furthermore, somatic mutations of the *APC* gene detected in several colorectal carcinomas, indicated involvement of the *APC* gene mutation in development of sporadic form of colorectal tumors (2). However, it is still uncertain (i) what proportion of sporadic tumors contain mutations of the *APC* gene, (ii) whether or not specific type of somatic mutations of the *APC* gene occur in tumors, (iii) whether or not somatic mutations occur in a particular 'hot spot' in the

APC gene, (iv) with which process(es) during carcinogenesis of colorectal tumors somatic mutations of the *APC* gene are associated, and (v) whether or not two hit mutations (4,5) of the *APC* gene are required for development of adenomas.

In order to address these questions, we have examined somatic mutation of the *APC* gene in colorectal tumors developed in FAP patients as well as sporadic form of adenomas and carcinomas, in addition to allelic loss at the APC locus. The results of genetic alterations reported here contribute significantly to understanding of tumorigenesis of colorectal adenomas and carcinomas.

RESULTS

Fig. 1 shows an example of RNase protection analysis in which variant patterns were detected in lanes 1 and 5. This results indicate sequence alterations in DNAs isolated from two adenomas A41 and A52. To characterize alterations observed by RNase protection analyses, DNA sequences of PCR products were determined as described in Materials and Methods. The result of DNA sequencing of the PCR product from a sporadic carcinoma C20 (lane 2 in Fig. 2 (A)) reveals a G to T change at the first nucleotide of codon 1286 resulting in a non-sense mutation from Glu (GAA) to a stop codon (TAA). Fig. 2 (B) and (C) show examples of insertion (lane 2) and deletion (lane 2) which were detected by extra bands (beginning at the arrow) following insertion or deletion of nucleotides. A 1-bp insertion (B) at codon 1287 (AATA) in a sporadic adenoma A53 and a 1-bp deletion (C) at codon 1439 (CCT) in a sporadic carcinoma C29 were recognized. The absence or significant reduction of normal sequences in three tumors shown in Fig. 2 indicated that the *APC* genes on both alleles were inactivated through loss of one allele and the mutation in the other allele.

Table 1 summarizes the results of 43 somatic mutations (not including LOH) observed in 63 colorectal tumors. All mutations were confirmed as somatic changes by a comparison with DNAs isolated from corresponding normal tissues. Twenty-one (49%) of them were point mutations; 16 were nonsense mutations resulting in truncation of the APC product. Two were missense mutations; from Glu to Gly in adenoma A128 and from Thr to Ala in adenoma A41. The other three point mutations occurred at splice acceptor or donor sites and at least two of them affected

the normal splicing of RNA. Among 21 point mutations, a C to T change was observed in eight cases (Table 2), five of which were at CpG sites.

As summarized in Table 3, nineteen somatic mutations were 1 to 31-bp deletions and three cases were 1-bp insertions. Except a 1-bp deletion observed in an intron (case C113), all of them

Figure 1. RNase protection analyses. Lanes 1 (sample A41) and 5 (A52) represent different abnormal patterns (arrows); RNA probe used corresponds to a region between codons 1389 and 1547.

altered the reading frame and created new stop codons at downstream. Among the 19 deletion cases, a 1-bp deletion was observed most frequently (nine cases).

The distribution of 43 somatic mutations (not including LOH) of the *APC* gene detected in colorectal tumors is shown in Fig. 3. Although somatic mutations were observed in exons 4, 5, 8, 9, 11, 12 and 15, more than three-quarters of them occurred in exon 15; fourteen of 21 point mutations (67%) and 19 of 22 (86%) deletions or insertions. Notably, 28 cases (65%) of total somatic mutations were clustered within a very small part (less than 10%) of the entire coding region from codon 1286 to 1513, which was designated as 'MCR' (mutation cluster region) of the *APC* gene in colorectal tumors. Mutations at seven specific sites were observed in more than one independent tumors (Table 1 and Fig. 3).

The number of genetic alterations in tumors

LOH was also examined as described in Materials and Methods. Combining three methods, all 63 tumors were informative and the loss of one allele at the APC locus was detected in 30 (48%) tumors. Table 4 summarizes the results of genetic alterations including LOHs, point mutations and frameshift mutations at the APC locus in 63 tumors. Nine (56%) of 16 adenomas contained two genetic alterations. In five (31%) adenomas, only one genetic change was observed and no alteration was detected in two adenomas. Among 47 carcinomas, two genetic alterations at the *APC* locus were found in 23 (49%) cases, one was detected in 16 (34%) cases, and no alteration was detected in 8 (17%) cases, respectively.

It is certain that the *APC* genes on both alleles were inactivated in 20 tumors among 32 tumors (nine adenomas and 23 carcinomas), in which two alterations were identified; in 19 tumors, one allele was lost and the other allele was mutated by a point mutation or a frameshift; in one sporadic carcinoma C27,
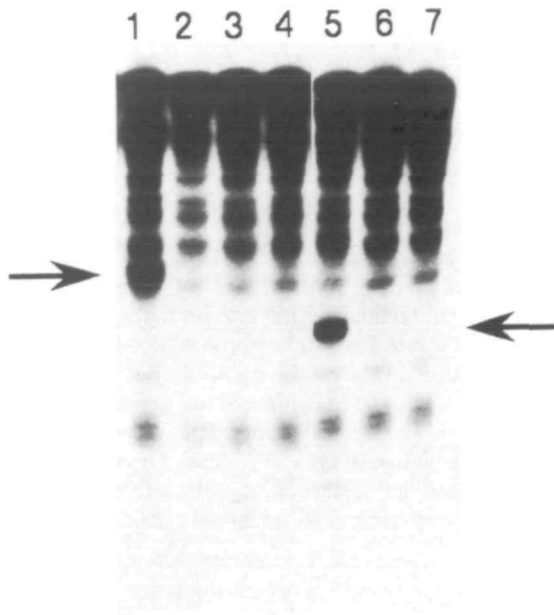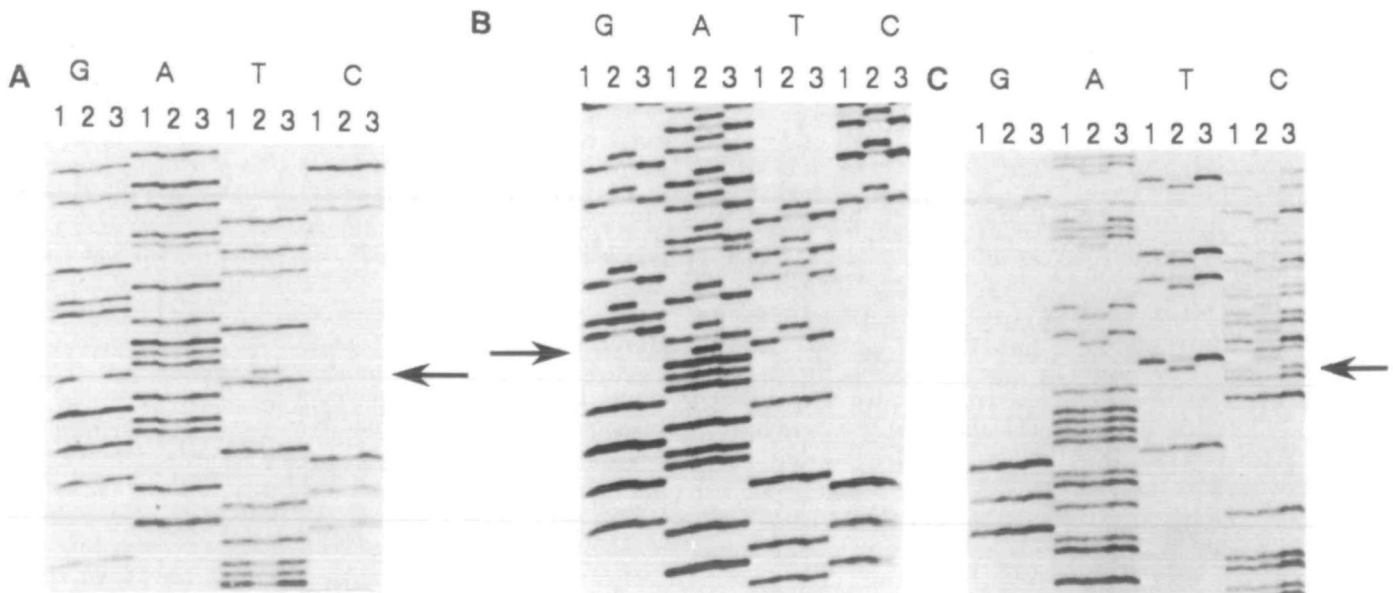
Figure 2. Sequence analyses of PCR products in three tumors. Sample C20 (lane 2 in (A)) shows a G to T change (arrow) at the first nucleotide of codon 1286 resulted in a change from Glu (GAA) to a stop codon (TAA). Sample A53 (lane 2 in (B)), an A insertion at the second nucleotide of codon 1287 (AATA) and sample C29 (lane 2 in (C)) shows a C deletion of codon 1439 (CCT) beginning at arrows. Extra bands can be seen after insertion or deletion in addition to faint bands, probably due to a contamination of normal cells.

one allele contained a 31-bp deletion beginning at codon 1293 and a nonsense mutation was found at codon 1367 in the other allele. Since codons 1293 and 1367 were contained in one PCR product, two alleles of different sizes could be easily separated by a polyacrylamide gel electrophoresis and each allele was independently sequenced (Fig. 4). Although two different somatic mutations of the *APC* gene were found 12 tumors (five adenomas and seven carcinomas), it is uncertain whether both alleles were inactivated or two somatic changes occurred in one allele.

Table 1. Forty-three somatic mutations of colorectal tumors in the APC gene

Tumors	Codon	Nucleotide Change	Effect of Mutation
C113(F)	142	aatag/GTC → atag/GTC	Splice acceptor
C31,C125(F)	213	CGA → TGA	Arg → Stop
C24	279	aattttag/GGT → agttttag/GGT	Splice acceptor
C47	298	CACTC → CTC	AC deletion
C108(F)	302	CGA → TGA	Arg → Stop
C135*	438	CAA/gtaa → CAA/gcaa	Splice donor
C33	516	AAG/gt → AAG/tt	Splice donor
C28	534	AAA → TAA	Lys → Stop
C10	540	TTA → TTAT	A insertion
C37	906	TCTG → TTG	C deletion
A128(F)	911	GAA → GGA	Glu → Gly
C23	1068	TCAAGGA → GGA	TCAA deletion
C11,C15	1114	CGA → TGA	Arg → Stop
C20	1286	GAA → TAA	Glu → Stop
A53	1287	ATA → AATA	A insertion
C27	1293	ACACAGGAAGCAGATTCTGCTAATACCC-TGCAAA → AAA	31 bp deletion
C7,C21	1309	GAAAAGAT → GAT	AAAGA deletion
C14	1309	GAA → TAA	Glu → Stop
A41	1313	ACT → GCT	Thr → Ala
C31,C42	1315	TCA → TAA	Ser → Stop
A44	1338	CAG → TAG	Gln → Stop
C22	1353	GAATTTTCTTC → TTC	8 bp deletion
A56	1356	TCA → TGA	Ser → Stop
C4,C27	1367	CAG → TAG	Gln → Stop
C10	1398	AGTCG → TCG	AG deletion
C19	1398	AGTC → AGC	T deletion
A43	1411	AGTG → ATG	G deletion
C16	1420	CCCA → CCA	C deletion
C40,A52(F)	1429	GAA → TAA	Gln → Stop
C29	1439	CCTC → CTC	C deletion
C37	1446	GCTCAAACCAAGC → GGC	10 bp deletion
A50(F)	1448	TTAT → TAT	T deletion
A49(F)	1465	AGTGG → TGG	AG deletion
C23	1490	CATT → CTT	A deletion
C12	1492	GCCA → GCA	C deletion
A41	1493	ACAGAAAGTACTCC → TCC	11 bp deletion
C3	1513	GAG → TGAG	T insertion

* Reported previously (2).

A and C show the histopathological type of tumors; adenoma and carcinoma, respectively.

(F) represent tumors derived from FAP patients and others were from non-FAP patients.

Table 2. Summary of somatic point mutations in the APC gene

From/To*	C	T	G	A	Total
C	–	8	1	2	11
T	1	–	0	0	1
G	0	5	–	0	5
A	0	1	3	–	4
Total	1	14	4	2	21

* Listed in coding strand.

DISCUSSION

We have characterized somatic mutations of the *APC* gene in a large number of colorectal tumors. Similar to germ-line mutations (3), the great majority (95%) of the somatic mutations observed in the *APC* gene led to truncation of the gene product (Table 3). Although it is uncertain that two missense mutations (Glu to Gly and Thr to Ala) significantly affect the biological activity of the APC protein, these mutations might imply the functionally important domains of the APC protein. Comparing somatic to germ-line mutations (3), the observed frequency of point mutations and frameshift mutations are similar (point mutation: 43% vs. frame shift mutations: 57% for germ-line and 49% vs. 51% for somatic mutations). Eleven (52%) of 21 point mutations occurred at C residues; most of them were at CpG or CpA sites (at five CpG and three CpA sites), similar to germ-line mutations (at seven CpA and five CpG sites among 23 point mutations) (3).
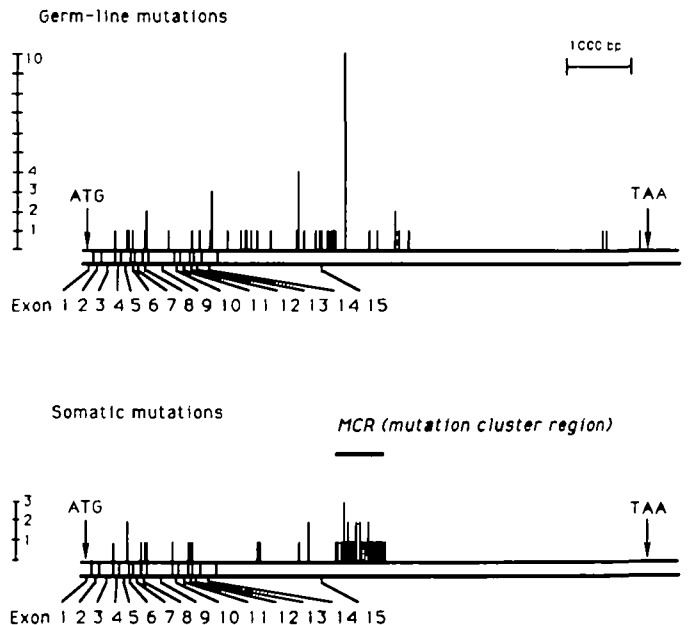

Figure 3. A comparison of distribution of germ-line mutations (3) and somatic mutations in the APC gene. The length of the bar indicates the number of FAP patients or colorectal tumors detected germ-line or somatic mutations at the indicated position. The positions of translational initiation (ATG) and termination (TAA) codons are marked with arrows.

Table 3. Frequency of somatic mutations in the APC gene

Point mutations		21
	Nonsense mutation	16
	Missense mutation	2
	Splice site	3
Frameshift mutations		22
	Deletion (1–31 bp)	19*
	Insertion (1 bp)	3
Total		43

* Including one deletion at the site of splicing.

Table 4. The result of genetic alterations at the APC locus observed in 63 colorectal tumors

Tumors	Genetic Alterations		Number of Alterations
ADENOMA			
A43	ST	LOH	2
A44	ST	LOH	2
A53	ST	LOH	2
A124(F)	GT	LOH	2
A41	ST	SM	2
A128(F)	GT	SM	2
A49(F)	GN	ST	2
A50(F)	GN	ST	2
A52(F)	GN	ST	2
A45	LOH		1
A51(F)	GN		1
A56	ST		1
A106(F)	GT		1
A114(F)	GT		1
A48			0
A54			0
CARCINOMA			
C3	ST	LOH	2
C4	ST	LOH	2
C7	ST	LOH	2
C11	ST	LOH	2
C12	ST	LOH	2
C14	ST	LOH	2
C15	ST	LOH	2
C16	ST	LOH	2
C19	ST	LOH	2
C20	ST	LOH	2
C28	ST	LOH	2
C29	ST	LOH	2
C24	SS	LOH	2
C135	SS	LOH	2
C107(F)	GT	LOH	2
C10	ST	ST	2
C23	ST	ST	2
C27	ST	ST	2
C31	ST	ST	2
C37	ST	ST	2
C108(F)	GT	ST	2
C125(F)	GT	ST	2
C113(F)	GT	SS	2
C1	LOH		1
C2	LOH		1
C5	LOH		1
C6	LOH		1
C8	LOH		1
C9	LOH		1
C13	LOH		1
C17	LOH		1
C18	LOH		1
C25	LOH		1
C21	ST		1
C22	ST		1
C40	ST		1
C42	ST		1
C47	ST		1
C33	SS		1
C26			0
C30			0
C32			0
C34			0
C36			0
C38			0
C39			0
C46			0

* Reported previously (2).
(F) represents tumors derived from FAP patients and others were from non-FAP patients. GT indicates a germ-line mutation resulting in truncation of the APC product; GN: a germ-line mutation was not determined. Germ-line mutations detected are: 2-bp deletions (codon 665) in A114 and C113; 5-bp deletions (857) in A124 and C125; nonsense mutation (1102) in A128; 5-bp deletions (1309) in A106, C107 and C108.
ST indicates a somatic mutation resulting in truncation caused by nonsense mutation, deletion or insertion; SM: missense mutation; SS: mutation at a splicing site.
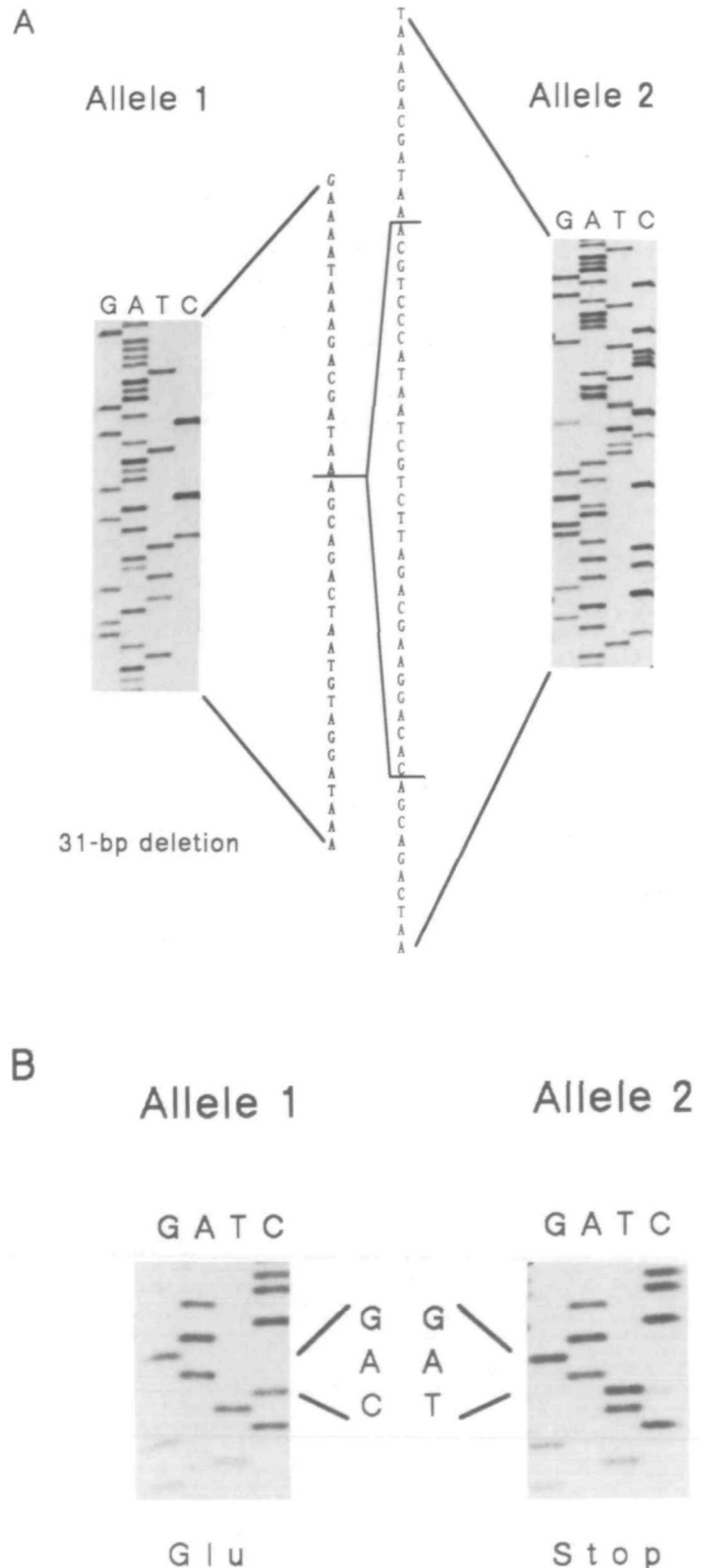LOH represents loss of heterozygosity.

A

Allele 1   Allele 2

31-bp deletion

B

Allele 1   Allele 2

Glu   Stop

Figure 4. DNA sequencing of the region (between codons 1260 and 1410) in a tumor T27. Two different size of the PCR products (a normal size of 449-bp and a shorter size of 418-bp) were separately subcloned and sequenced. Allele 1 of this tumor (418-bp fragment) reveals a 31-bp deletion that begun at codon 1293. Allele 2 (449-bp) contains a mutation from C to T at codon 1367 (449-bp fragment) resulting in creation of a stop codon.

Although somatic mutations were observed in a large portion of the *APC* gene from codon 142 to 1513, 28 (65%) of 43 mutations were clustered within a small region (less than 10% of the coding region) codon 1286 to 1513, designated as 'MCR' (mutation cluster region). Germ-line mutations in more than 90 FAP patients were also commonly found in this region but the frequency was only 23% (3 and unpublished data).

More than half of 63 colorectal tumors had two genetic alterations at the APC locus and in the remainder we identified a single or no alteration. In this study, no significant difference was observed between adenomas and carcinomas. These results support the hypothesis that the *APC* gene mutation is associated with the early stage of the carcinogenesis of colorectal tumor (10). However, it is notable that the frequency of somatic mutations of the *APC* gene in this study is likely to be underestimated because (i) RNase protection analysis does not detect all mismatches and (ii) we have not examined introns or 5' flanking region of the *APC* gene. In our previous study of a substantial number of FAP patients using the same method, we found germ-line mutations in only 70% of patients (3). Hence, somatic mutations of the *APC* gene should be higher than we reported here, suggesting that the mutations of the *APC* gene, probably on both alleles, are involved in the great majority of colorectal tumors.

MATERIALS AND METHODS

Genomic DNA of colorectal tumors

Genomic DNA from 63 colorectal tumors, including eight adenomas and four carcinomas developed in five FAP patients as well as eight adenomas and 43 carcinomas in 48 non-FAP patients, were prepared from specimens resected during the course of surgical or fiberscopic treatments. DNA from their corresponding normal mucosa or peripheral white blood cells was also prepared as described elsewhere (6). All adenomas were larger than 1 cm and histopathological diagnoses were performed for all samples.

PCR

Thirty-one segments of the entire coding region of the *APC* gene were separately amplified by a polymerase chain reaction (PCR) method. The primer pairs used in this study were described previously (3). PCR was performed with 40 cycles consisting of 30 sec. at 95°C, 2 min. at 51°C and 2 min. at 70°C as described by Baker et al. (7).

RNase Protection Analysis

To screen an alteration of DNA sequence, RNase protection analysis coupled with PCR technique was performed according to the method of Winter et al.(8) as modified by Kinzler et al. (1). PCR products were digested with RNase A, which cleaved RNA at mismatches within DNA-RNA hybrids, after hybridization to ^{32}P-labeled RNA transcripts corresponding to normal *APC* sequences and then analyzed by a polyacrylamide gel electrophoresis. Both strands of RNA transcripts were separately used for RNase protection analyses.

Cloning and Sequence Analysis

PCR products which showed a different pattern by RNase protection analyses, were cloned into a plasmid vector (pBluescript SK Stratagene), as described (2). Template DNA was prepared from more than 100 independent subclones for DNA sequencing as described by Nigro et al.(9).

Detection of loss of heterozygosity (LOH)

LOH was examined by one or more of the following methods; (i) normal and tumor DNAs were compared by Southern hybridization using polymorphic cDNA clones of the *APC* gene as a probe (unpublished data). (ii) Following PCR, a RsaI polymorphism at codon 486 (TAC/TAT) in exon 11 of the *APC* gene was identified (3). Primers used for PCR were 5'-TAGATGATTGTCTTTTTCCT-CT-3' and 5'-TCATACCTGAGCTATCTTAAG-3'. (iii) DNA sequence poly-morphism in the exons of *APC* gene (3) was used for a comparison between normal and tumor DNAs.

ABBREVIATIONS

APC, adenomatous polyposis coli.
FAP, familial adenomatous polyposis.
LOH, loss of heterozygosity.
MCR, mutation cluster region.

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