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Advances in Brief

Coexistence of Somatic and Germ-Line Mutations of *APC* Gene in Desmoid Tumors from Patients with Familial Adenomatous Polyposis[1]

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Abstract

Desmoid tumors, which are locally invasive with recurrence but without metastasis, are frequently observed in patients with familial adenomatous polyposis after abdominal surgery or during pregnancy. This study analyzed mutation of the adenomatous polyposis coli gene in 8 desmoid tumors from 7 familal adenomatous polyposis patients using polymerase chain reaction-single-strand conformation polymorphism and the direct sequencing method. Seven somatic mutations, 1 somatic allele loss, and 6 germ-line mutations were detected. The majority of adenomatous polyposis coli gene mutations were deletions of 1 to 19 base pairs in exon 15, and all mutations led to the formation of stop codons. A somatic mutation with repetition of 82 base pairs from codon 1399 to 1426 was also observed in a desmoid, which was most likely caused by an error during replication or repair replication. No mutation was detected in exons 1 to 2 of H-*ras*, K-*ras*, and N-*ras* genes and in exons 5 to 8 of *p53* gene, in these tumors. The simultaneous existence of somatic and germ-line alterations of adenomatous polyposis coli gene observed in all 8 tumors strongly suggests that inactivation of both alleles of adenomatous polyposis coli gene is involved in the development of desmoid tumors.

Introduction

Although FAP[3] is characterized by numerous colorectal adenomas, it is also recognized as a growth disorder affecting the whole body (1). Extracolonic manifestations include gastric and duodenal polyps, desmoid tumors, osteoma, thyroid tumors, adrenocortical tumors, brain tumors, retinal pigmentation, and tumors in other organs. Some of these benign tumors become malignant if left untreated. A high incidence of desmoid tumors has been observed in FAP patients, as high as 8–12% (2), most of which arise during the 2 years following abdominal surgery. These desmoids grow to a very large size and often recur many times but without metastasis.

FAP is a dominant trait that is determined by the heterozygous mutant *APC* gene. We have previously observed the LOH at the chromosome 5q21–22 region which includes the *APC* gene, in both a recurring desmoid tumor (3, 4) and an adrenocortical carcinoma (5) in FAP patients, as well as a high frequency of LOH in colorectal tumors (6, 7). The loss of the chromosomal region that includes the normal allele of the *APC* gene could be predicted by genetic analysis of family members in both cases, which leads to the assumption that the inactivation of both alleles of the *APC* gene is involved not only in the development of colorectal tumors but also extracolonic tumors. The *APC* gene was isolated and the sequence of its complementary DNA was determined in 1991 (8–11), therefore genetic changes can now be analyzed in tumors even without LOH. In the present investigation we found the occurrence of not only germ-line but also somatic mutations in 8 desmoid tumors that developed in FAP patients after colectomy and during pregnancy.

Materials and Methods

Desmoid Tumors. Eight desmoid tumors were obtained from 7 FAP patients and histopathologically diagnosed as desmoid. Clinical data of individual tumors are indicated in Table 1.

PCR-SSCP Analysis. DNA was extracted from each desmoid tumor and the corresponding normal tissue or peripheral blood using pronase K, sodium dodecyl sulfate, and phenol-chloroform. DNA samples were amplified for SSCP analysis of *APC* gene using PCR (5 min at 97°C, 1 time; 1 min at 95°C, 1 min at 58°C or 60°C, and 1 min at 72°C for 35 or 50 cycles; and 10 min at 72°C, 1 time). The reaction mixture (5 μl) contained 200 ng of genomic DNA, the proper pair of each 0.2 μM primer, 25 μM each deoxynucleoside triphosphate, 1× PCR buffer, Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT), and [α-^{32}P]dCTP. Oligodeoxynucleotide primers used to amplify the 15 coding exons of *APC* gene, including 76 primers for 38 regions, were the same as those reported (8). Primers to amplify the exons 5 to 8 of *p53* gene and conditions for PCR were those previously reported (12). Primers used to amplify exons 1 and 2 of H-*ras*, K-*ras*, and N-*ras* genes were from Takara Biochemicals (Kyoto). PCR products were diluted 10-fold with formamide-dye solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), and a 2-μl sample of the diluted reaction mixture was heated for 5 min at 80°C followed by electrophoresis in 5% polyacrylamide gel containing 5% glycerol, as described (13). After electrophoresis at 20–25°C, gel was exposed to x-ray film at −70°C. PCR was performed at least twice for each sample and only the reproducible cases were taken.

Sequencing of the Mutated Strand. Abnormal single-stranded DNA fragments were extracted with distilled water from the corresponding bands on PCR-SSCP gels as described (14). The DNA fragments were amplified through the asymmetrical PCR (15) in 100 μl of mixture under the same conditions as those for PCR-SSCP analysis, with the exception that the ratio of primers was 100/1 or 1/100 for sense and antisense primers. The amplified DNA samples were purified using QIAGEN spin 20 column (Qiagen Inc., Chatsworth, CA) and sequenced with the dideoxy chain-termination reaction using Sequenase Version 2.0 (United States Biochemical Co., Cleveland, OH). Primers used for sequencing were the same as those in PCR-SSCP. Sequencing was performed more than twice for each DNA fragment.

Results

Clinical data regarding the 8 desmoid tumors of the present study are shown in Table 1. Five desmoids were in abdominal wall and 3 were in the mesentery, 5 of which developed 6 to 36 months after abdominal surgery. One desmoid was newly formed 10 months after surgical removal of the first desmoid. The size of 5 tumors was 2–10 cm in maximum diameter, and 3 tumors had grown to weigh as much as ~1700 g. Recurrence was observed in 4 cases, although none of these desmoids was tumorigenic in nude mice.

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[3] The abbreviations used are: FAP, familial adenomatous polyposis; *APC*, adenomatous polyposis coli; LOH, loss of heterozygosity; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism.

Table 1 *Clinical data of desmoid tumors from FAP patients*

Tumor	Site	Size[a]	Recurrence	Sex	Age[b]	Other manifestations
PLK42-Desmoid	Mesentery (after pregnancy)	450–1700g	+ (4 times)	F	29	Colorectal polyps
PLK56-Desmoid	Abdominal wall (during pregnancy)	9 × 4 × 4 cm	+ (1 time)	F	30	Colorectal polyps, Gastric polyps, Thyroid tumor, CHRPE[c]
PLK59-Desmoid	Abdominal wall (36 m after colectomy)	6 × 3 cm	+ (1 time)	M	51	Colorectal polyps, gastric polyps, CHRPE
PLK111-Desmoid	Mesentery (after colectomy)	1000 g	–[d]	M	30	Colorectal polyps
PLK124-Desmoid	Abdominal wall (18 m after colectomy)	5 × 8 × 4 cm	+ 3 (times)	F	21	Colorectal polyps, Duodenal polyps, Osteoma
PLK126-Desmoid	Mesentery (12 m after colectomy)	10 × 10 cm	–	F	21	Colorectal polyps and cancer, CHRPE
PLK150-Desmoid	Abdominal wall (6 m after colectomy)	1400 g	–	F	27	Colorectal polyps, Duodenal polyps, CHRPE
PLK150-Desmoid-R	Abdominal wall (10 m after removal of PLK150-Desmoid)	2 × 2 × 2 cm	–	F	28	

[a] Size or weight of tumor which was analyzed for mutation.
[b] Age of patient at removal of the primary desmoid.
[c] CHRPE, congenital hypertrophy of the retinal pigment epithelium.
[d] –, no recurrence or unknown.

DNAs from both desmoid tumors and corresponding normal tissues were analyzed for mutation in exons 1 to 15 of *APC* gene using the PCR-SSCP method. Examples are shown in Fig. 1, *A* and *B*. Electrophoresis of the denatured PCR products of exon 15-E detected two abnormal bands, in addition to the two normal bands, in the cases of FAP patients PLK56, PLK59, and PLK111 (Fig. 1*A*). The appearance of abnormal bands suggested the presence of mutation in the 15-E sequence in one allele of the *APC* gene pair. These mutations were considered to be germ-line mutations, since the same abnormal bands were seen in both desmoid DNA and normal DNA from the same patients. On the other hand, PCR-SSCP analysis of exon 15-H in Fig. 1*B* detected abnormal bands only in the desmoid DNAs from FAP patients PLK56, PLK111, PLK126, and PLK150, which indicated that these bands were derived from somatic mutations that existed only in the desmoid tumors.

In FAP patient PLK42 (Fig. 1*B*), the abnormal bands for germ-line mutation of exon 15-H were seen in both DNAs from desmoid and normal tissues, but normal bands were detected in DNA from only normal tissue. This pattern indicates that the normal allele of *APC* gene in this desmoid had been lost, which has previously been suggested from the genetic analysis of LOH in the family members of this patient (3).

All SSCP patterns in the present analysis were reproducible. DNA fragments in the abnormal bands were reamplified by asymmetrical PCR and then sequenced, and an example of the sequencing is shown in Fig. 1*C*.

Six germ-line mutations and 7 somatic mutations were determined in the regions that included exons 15-C, 15-E, 15-G, 15-H, and 15-I of *APC* gene, as listed in Table 2. Three germ-line mutations existed close together at codons 1061, 1105, and 1110 in 15-E in PLK59, PLK56, and PLK111, where somatic mutation was not detected. Both germ-line mutations at 1061 and 1105 led to the formation of a stop codon (TAA) at 1125–1126. The 5-base deletion (ACAAA) at codon 1062–1063 has been frequently observed as a germ-line mutation in FAP families (16, 17), but the germ-line mutation of PLK59 was a 4-base deletion (AACA). Germ-line mutation in PLK126 was A to T transversion at 848, and that in PLK111 was C to G transversion at 1110. A germ-line mutation of a 5-base deletion at codon 1309–1311 of 15-G in PLK124 led to a stop codon at 1313–1314 (TAG).

Around the codons from 1452 to 1470 of 15-H, six mutations were detected: one germ-line mutation in PLK42; and five somatic mutations in desmoid tumors from patients PLK56, PLK124, PLK126, and PLK150. Each case had a different pattern of deletion that included 1–19-base deletions, but 5 of the 6 different mutations produced the same stop codon (TAA) at downstream codon 1472–1473. PLK150-Desmoid and PLK150-Desmoid-R from the same patient had different somatic mutations at codons 1458 and 1470, respectively. The mutant bands observed in PLK150-Desmoid were not detected in PLK150-Desmoid-R in the PCR-SSCP analysis, which indicated that these two desmoids had originated from different cells. The somatic mutation in PLK59-Desmoid, occurring at codon 1581–1584 with an 8-base deletion (TGCCATGC), produced a stop codon (TAA) at 1589–1590.

The somatic mutation in the desmoid tumor from PLK111 was found to include the repetition of 82 base pairs from codon 1399 to 1426 in exon 15-H. Such an insertion of a long sequence exhibited abnormal bands that moved much slower than normal bands in PCR-SSCP (Fig. 1*B*). The insertion of the 82 base pairs between the first A and the second G of codon 1426 caused the formation of a stop codon (TGA) at 1407–1408 in the inserted sequence, as indicated in the lower part of Table 2. Such a type of somatic mutation has not previously been reported in the *APC* gene. The slowly electrophoresed abnormal bands were also detected in this tumor when another PCR primer set was used (data not shown).

The 8 desmoids analyzed had both somatic and germ-line alterations of the *APC* gene, although germ-line mutations in a FAP patient PLK150 has not yet been determined. None of the 8 desmoids had more than 2 mutations within exons 1 to 15 of the *APC* gene.

Since K-*ras* gene and *p53* gene mutations have frequently been detected in colorectal tumors from FAP patients (7, 12), examination of mutations of exons 1 and 2 of H-*ras*, K-*ras*, and N-*ras* genes and of exons 5 to 8 of *p53* gene, in the desmoid tumors, was done using PCR-SSCP method. However, no mutation was detected for these genes in any of the 8 desmoid tumors.

Discussion

All of the 8 desmoid tumors analyzed in this study had somatic alterations in *APC* gene: 7 had mutations and 1 had loss of normal allele, in addition to the germ-line mutations. Also all mutations led to stop codons, which result in the synthesis of truncated APC proteins. The simultaneous existence of two genetic alterations in individual desmoids suggests that both alleles of *APC* gene were inactivated in these tumors, although it is not yet fully confirmed that the mutations

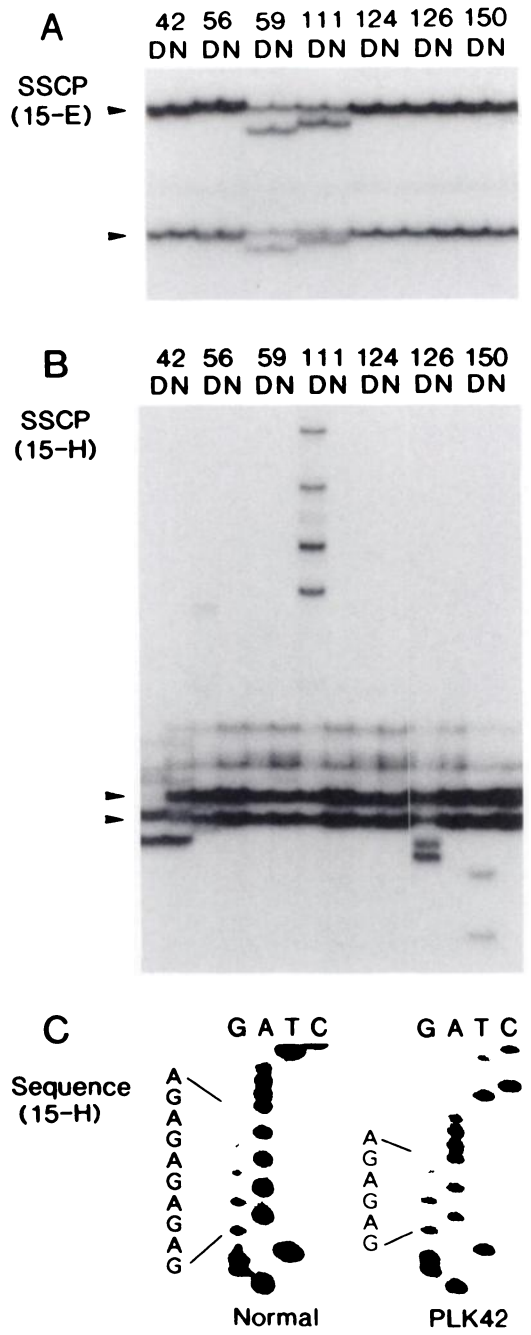

Fig. 1. PCR-SSCP and direct sequencing of *APC* gene in DNAs of desmoid tumors and normal tissues from FAP patients. Specific sequences of *APC* gene were amplified from genomic DNAs from desmoid tumors and normal tissues using PCR, and denatured PCR products were separated electrophoretically followed by the direct sequencing as described in "Materials and Methods." *A*, PCR-SSCP patterns in exon 15-E. *B*, PCR-SSCP petters in exon 15-H. D, desmoid; N, normal tissue; number is individual FAP patient; *Arrows*, normal bands. *C*, sequencing of DNA fragments eluted from the normal and abnormal PCR-SSCP bands in exon 15-H. In *A*, germ-line mutations are seen in both desmoid and normal tissues from PLK56, PLK59, and PLK111. In *B*, germ-line mutation is seen in both desmoid and normal tissue of PLK42, and somatic mutations are seen in desmoids from PLK56, PLK111, PLK126, and in PLK150-desmoid.

exist on both alleles. In PLK42-Desmoid, it was clear that somatic allele loss and germ-line mutation existed in the two different *APC* alleles.

The cause of the production of somatic mutations is still unclear, but it can be assumed from the nature of mutations. The current study observed that almost all somatic and germ-line mutations in desmoid tumors are of the same nature, in that the deletions occur between or within areas of repeated sequences of exon 15, except for two germ-line mutations with one base change. In the region from 1458 to 1465, four deletions were detected as follows: 2-base deletion at 1461–1462 CCTACTGCTGA(AA)AGAGAGAGAG; 4-base deletion at 1462–1465 CCTACTGCTGAAAAGAG(AGAG)AG; 10-base deletion at 1458–1461 CC(TACTGCTGAA)AAGAGAGAGAG; and 19-base deletion at 1458–1464 CC(TACTGCTGAAAAGAGAGAG)AG.

Somatic mutation of a 1-base deletion at 1470 GC(T)GC, somatic mutation of an 8-base deletion at 1581–1584 ATTATTTC(TGCCATGC)CAACAA, and germ-line mutations of a 1-base deletion at 1105–1106 GG(G)G, a 4-base deletion at 1061–1063 AA(AACA)AA, and a 5-base deletion at 1309–1311 AAAAGA(AAAGA)TTGGAA, were also between repeated sequences. The 82-base repeat from codon 1399 to 1426 of the *APC* sequence in PLK111-Desmoid strongly suggests that this novel genetic alteration occurred due to a DNA replication error. These somatic mutations occurring in desmoid tumors appear to be caused by misalignment of the DNA strands during replication in the proliferating cells after abdominal surgery or by repair replication after incision of DNA in musculoaponeurotic soft tissue. It should be noted that the second desmoid tumor PLK150-Desmoid-R, formed 10 months after surgical removal of the first tumor 150-Desmoid, had a somatic mutation which was different from the mutation in the first desmoid. This fact suggests that the somatic mutation of the *APC* gene in the second desmoid newly occurred after removal of the first desmoid.

In contrast to such a predominance of microdeletions in the *APC* gene in desmoids, point mutations, which appeared to be mainly caused by mutagens, were dominant in the *p53* gene in colorectal tumors from FAP patients, as has been previously described (12). This difference in the characteristic of mutation suggests a difference in the contribution of environmental mutagens or carcinogens between *APC* mutation and *p53* mutation. It can be presumed that the major cause of *APC* mutation is replication error and that of *p53* mutation is DNA lesion by mutagens.

Although there are many repeated sequences in the *APC* gene, the region where mutations are frequently detected appears to be not only in the most easily mutable sequence, but also in the important regions with regard to the development of tumors or loss of function of APC protein. The region from codon 1452 to 1470, which frequently experiences somatic mutation in desmoid tumors, falls within the region where somatic mutations have frequently been detected in colorectal tumors (19, 20). Mutation at codon 1309–1311 has been the most frequent germ-line mutation in FAP patients previously described (16, 17, 18), and we also detected the same mutation in PLK124 and another 15 independent FAP families.[4] The mutation at codon 1062 has been assumed to be the second most frequent germ-line mutation (16, 17), and around this codon three germ-line mutations were also observed in the present study.

The absence of mutations in H-*ras*, K-*ras*, and N-*ras* genes and in *p53* gene in these tumors suggests that inactivation of the *APC* gene is significant for induction of the aggressive growth of desmoid tumors of frequent recurrence. This fact appears to be an important clue to the molecular mechanisms of desmoid formation. Desmoid tumors are defined as benign fibrous tumor which do not metastasize, and it is reasonable to assume that *p53* mutation is absent, since *p53* mutation is usually involved in the change from benign to malignant nature, in colorectal tumors (12). Furthermore, it has been assumed that K-*ras* mutation contributes to rapid growth of both benign and malignant colorectal tumors because of the greater frequency of K-*ras* mutation in large polyps in FAP patients (7). However, despite the lack of any *ras* mutation, some desmoid tumors grow very rapidly. It is necessary to determine if inactivation of the *APC* gene is the only

[4] Unpublished data.

Table 2 *Germ-line and somatic mutations of the APC gene in desmoid tumors from FAP patients*[a]

Tumor	Germ-line mutation		Somatic mutation	
	Codon	Mutation	Codon	Mutation
PLK42-Desmoid	1462–1465	(AGAG) del	Loss of normal allele[b]	
PLK56-Desmoid	1105–1106	(G) del	1461–1462	(AA) del
PLK59-Desmoid	1061–1063	(AACA) del	1581–1584	(TGCCATGC) del
PLK111-Desmoid	1110	T(C)A→T(G)A	1399–1426	(C····A) 82-base pair repeat[c]
PLK124-Desmoid	1309–1311	(AAAGA) del	1452	(G) del
PLK126-Desmoid	848	(A)AA→(T)AA	1458–1561	(TACTGCTGAA) del
PLK150-Desmoid			1458–1464	(TACTGCTGAAAAGAGAGAG) del
PLK150-Desmoid-R			1470	(T) del

[a] All mutations led to stop codons.
[b] The loss was detected both in primary and recurring desmoid.
[c] Sequence of somatic mutation, which includes the 82-base pair repeat from codon 1399 to 1426, in PLK111-Desmoid is as follows:

1399 1426
····· CGT TCG ATT GCC AGC TCC GTT CAG AGT GAA CCA TGC AGT GGA ATG GTA AGT GGC ATT ATA AGC CCC AGT GAT CTT CCA GAT A
1399 1407 1408 1426
(CGT TCG ATT GCC AGC TCC GTT CAG AGT GAA CCA TGC AGT GGA ATG GTA AGT GGC ATT ATA AGC CCC AGT GAT CTT CCA GAT A)GC ·····

cause of such aggressive growth. Moreover, desmoid tumors contain a significant amount of fibrous components, and the mechanism of the increased production of fiber or collagen should also be examined.

The present finding of two-hit inactivation of the *APC* gene in desmoid tumors is significant for understanding the mechanism of extracolonic manifestations and also for understanding the function of the *APC* gene as a tumor suppressor.

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