p53 Gene Mutations in Colorectal Tumors from Patients with Familial Polyposis Coli

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ABSTRACT

The p53 gene has been elucidated as a tumor suppressor gene, and inactivation of this gene caused by deletion or point mutations may play a crucial role in the development of human malignancies. In colorectal carcinomas with an allelic deletion of the p53 gene, the remaining p53 gene was mutated with considerable frequency. It is most difficult to detect point mutations or small deletions of the gene because the mutations occur in diverse regions; although four hot spots have been observed [J. M. Nigro et al., Nature (Lond.), 342:705–708, 1989]. The polymerase chain reaction and denaturing gradient gel electrophoresis facilitate detection of mutations in the hot spots of the p53 gene. Using these methods, we detected mutations in three adenomatous polyps and one carcinoma from familial polyposis coli patients and three carcinomas of sporadic cases. The DNA sequence analysis confirmed mutations of the p53 gene in 2 adenomas (13 base-pair deletions in one and a point mutation in the other) and 1 carcinoma (point mutation) from familial polyposis coli patients. These results suggest that the p53 gene mutations may be involved in the formation not only of carcinomas but also of adenomas which occur in familial polyposis coli patients.

INTRODUCTION

Familial polyposis coli is a single autosomal dominant disorder characterized by the onset of numerous colorectal adenomas early in life and progression to adenocarcinoma in almost 100% of untreated patients. In general, adenomatous polyps in the colon and rectum are thought to progress to adenocarcinoma not only in FPC but also in cases of NPCC. Therefore, FPC provides an excellent model for molecular analysis of oncogenesis, in which the accumulation of genetic alterations of both DNA and chromosomes in a single cell is involved. In colorectal carcinomas, genetic alterations include the activation of the K-ras gene (1–3), and allelic losses of chromosomes 5q, 14, 17p, 18q, and 22 (3–6) and deregulated expression of the c-myc and c-fos genes are observed (7, 8). Allelic losses have been regarded as evidence that the affected regions contain a tumor suppressor gene or genes (9, 10). The gene responsible for FPC (FPC gene) was localized on chromosome 5q by linkage studies (11, 12), and frequent allelic loss of chromosome 17p, since mutation of the remaining p53 gene was a frequent occurrence (17, 18).

p53 was first identified as a cellular protein present in complex with the SV40 early-region encoded T-antigen protein (19, 20). Although the p53 gene was originally considered to be an oncogene, several recent reports have indicated that the wild-type gene product actually functions as a tumor suppressor gene (21, 22). Its mutations are clustered in four hot spots which coincide exactly with the most highly conserved regions of the gene among species (17). However, several mutations were found in different regions; therefore it is not easy to detect the point mutations or small deletions of this gene. To search for the mutations in this gene, we established the combined method of PCR and denaturing gradient gel electrophoresis (23). PCR products about 300–500 base pairs long, differing by single base-pair substitutions or small deletions, were clearly separated in the denaturing gel. Using these methods, we examined the p53 gene mutations in colorectal carcinomas from patients with FPC.

MATERIALS AND METHODS

Materials. We analyzed 75 primary colorectal tumor specimens from 36 Japanese patients with FPC and 15 with NPCC. There were 45 adenomas from 34 patients with FPC, 15 carcinomas from 14 patients with FPC, and 15 carcinomas from 15 patients with NPCC. In addition to primary tumors, 3 kinds of carcinomas from 3 independent patients with FPC transplanted into nude mice were also examined. Corresponding normal colonic mucosa were obtained from each patient. All of these specimens were obtained at the time of surgery and were snap frozen in liquid nitrogen.

DNA Preparation. High-molecular-weight DNA was isolated from surgical tumor specimens and/or their transplants into nude mice and the corresponding normal mucosa by the method of SDS-protease K and phenol/chloroform treatment (24).

RNA Preparation. RNA was extracted by the phenol/SDS method from 3 kinds of tumors transplanted into nude mice and separated from DNA by selective participation using LiCl, as described by Ausbel et al. (25).

p53 cDNA Synthesis, Amplification, and Sequence. First-strand cDNA was generated from 10 μg of total RNA with p53-specific oligonucleotide primer P1 (50 pmol) in the presence of reverse transcriptase at 42°C for 60 min in a 20-μl reaction (26). The consecutive amplification of the p53 cDNA was performed by PCR, as described (27), using Taq polymerase instead of a Klenow fragment. The reaction was performed in a total volume of 50 μl with 10 μl of the cDNA solution described above; primers P1 and P2 at 100 pmol each in 42 mM KCl; 10 mM Tris, pH 8.3; 2.5 mM MgCl2; 0.01% gelatin; dATP, dCTP, dGTP, and dTTP at 0.2 mM each; and 2 units of Taq polymerase for 33 cycles of 95°C denaturation (1 min), 55°C annealing (30 s), and 72°C extension (2 min) in a programmable heat block (DNA thermal cycle). The PCR products contained codons 122–393 of p53 cDNA. The primers used for the reaction were P1, 5′GGTTGTACCCCTGTCAGTTGGGGAACAA3′, and P2, 5′GCCAAGCTTGTGACTGGGCGCGTACT3′. Primers P3 and P4 had extraneous nucleotides comprising KpnI and HindIII sites, respectively, at their 5′ ends to facilitate cloning. The PCR products were digested with KpnI and HindIII, fractionated by electrophoresis, and ligated to KpnI- and HindIII-C

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2 To whom requests for reprints should be addressed.

The abbreviations used are: FPC, familial polyposis coli; NPCC, nonpolyposis colorectal cancer; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; cDNA, complementary DNA.
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**HindIII-digested Bluescript vectors (Stratagene). DNA sequence analysis** was performed according to the dideoxy chain termination method (28).

DNA Amplification. Total genomic DNA (0.1 μg) was used in four separate 50-μl PCR reactions for 30 cycles (96°C for 1 min, 60°C for 30 s, 72°C for 1 min) to generate fragments corresponding to exons 5 and 6 and intron 5 (primer set 2), exon 7 and intron 7 (primer set 3), exons 8 and 9 and intron 8 (primer set 4), and exons 5, 6, and 7 and introns 5 and 6 (primer set 5) (primer set 2: P5, 5’ TTCTCTGAGTACTCCTCGACAGACCTCCAGAGGGCGGC3’; set 3: P6, 5’TCTCTGAGTACTCCTCGACAGACCTCCAGAGGGCGGC3’; set 4: P10, 5’TTCTCTGAGTACTCCTCGACAGACCTCCAGAGGGCGGC3’; and P11, 5’CAGGGATCCCAAAGCTGTTCCGTCCCA3’; set 5: P13, 5’TCTCCTAGGTTGGCTCTG3’, and P11, 5’AGTGGATCCTGACAGACCTCAGGCGGCS’; introns 5 and 6 (primer set 5) (primer set 2: P5, 5’TTCCTGCAGTACTCCTCGACAGACCTCCAGAGGGCGGC3’; set 4: P10, 5’TTCTCTGAGTACTCCTCGACAGACCTCCAGAGGGCGGC3’; and P11, 5’CAGGGATCCCAAAGCTGTTCCGTCCCA3’; set 5: P13, 5’TCTCCTAGGTTGGCTCTG3’, and P11, 5’AGTGGATCCTGACAGACCTCAGGCGGCS’).

All primers except P3 were located on the exon-intron junction. Primer P13 was located in exon 8. In the PCR reactions, primer sets 2, 3, and 4 generated 402-, 535-, and 333-base pair fragments, respectively. The PCR product generated by primer set 5, primers P5 and P9, which had extraneous nucleotides comprising PstI and BamHI sites, respectively, at their 5’ ends, were cloned into the Bluescript vector and sequenced as described for p53 cDNA.

**Dot Blot Hybridization.** Oligonucleotide probes specific for normal and mutated sequences at codon 215 of the p53 gene were 5’TTCGACATAGTGTGGTG3’ and 5’TTCGACATGGTGTG-3’ respectively (Fig. 2). The probes were end-labeled with [7-32P] ATP in the presence of T4 polynucleotide kinase. Amplified DNA fragments (3 μl) generated by primer set 2 were dot blotted onto nylon membrane (Hybond N+) and hybridized with radiolabeled oligomer probes. The filters were prehybridized for 4 h at 54°C in solution A (3.0 M tetramethylammonium chloride-50 mM Tris-HCl, pH 8.0-2 mM EDTA-0.1% SDS-5 x Denhardt’s solution-100 μg/ml denatured herring sperm DNA) and hybridized with radiolabeled oligomer probes. The filters were prehybridized for 4 h at 54°C in solution A (3.0 M tetramethylammonium chloride-50 mM Tris-HCl, pH 8.0-2 mM EDTA-0.1% SDS-5 x Denhardt’s solution-100 μg/ml denatured herring sperm DNA) and hybridized with radiolabeled oligomer probes. The filters were prehybridized for 4 h at 54°C in solution A (3.0 M tetramethylammonium chloride-50 mM Tris-HCl, pH 8.0-2 mM EDTA-0.1% SDS-5 x Denhardt’s solution-100 μg/ml denatured herring sperm DNA) and hybridized with radiolabeled oligomer probes.

**Restriction Fragment Length Polymorphism Analysis.** Genomic DNA (8 μg) from 75 tumors and corresponding normal tissues was digested with BanI and hybridized with p53 cDNA as a probe, as described by Masharani et al. (29). Electrophoresis, transfer, and hybridization were performed as described (6).

**Denaturing Gradient Gel Electrophoresis.** The polyacrylamide concentration (120 mg/ml; acrylamide:bisacrylamide, 29:1) and the buffer concentration (120 mg/ml; acrylamide:bisacrylamide, 30:1) and the buffer concentration (120 mg/ml; acrylamide:bisacrylamide, 30:1) were uniform throughout the gels containing a linear gradient of formamide uniform throughout the gels containing a linear gradient of formamide uniform throughout the gels containing a linear gradient of formamide uniform throughout the gels containing a linear gradient of formamide.

**RESULTS**

**Analysis of p53 Gene Mutations by PCR and DNA Sequencing in the Remaining Allele.** We reported that the loss of heterozygosity on chromosome 17 in carcinomas from patients with FPC or NPCC occurred at rates of 31 and 27%, respectively (6). To determine whether the remaining allele in carcinoma from FPC had mutations in the p53 gene, we analyzed a carcinoma from KUPL40, one of the FPC patients. The carcinoma showed an allele loss for D17S5, on the short arm of chromosome 17 where the p53 gene was mapped, and the related tumors transplanted into nude mice were used for examination. Although primary tumors contain nonneoplastic cells, tumors transplanted into nude mice carried none of these cells. The p53 cDNA generated by primer set 1, including codons 122-393 (Fig. 1), were cloned and sequenced as described in “Materials and Methods.” In the transplanted tumor from KUPL40, we found one nucleotide change, when a comparison was made with p53 cDNA sequence (30). A transition from A to G had occurred within codon 215 (AGT to GGT), in the result being a change of the encoded amino acid from serine to glycine. We also examined two other transplanted tumors without an allelic loss of chromosome 17 but found no mutations. To determine whether the sequence change was derived from a somatic mutation and whether the same mutation occurred in the primary carcinoma and other tumors from FPC and NPCC, PCR was used to amplify a 402-base pair segment surrounding the presumptive mutation from total genomic DNA (0.1 μg) of 78 tumors, including 3 tumors transplanted into nude mice and 51 corresponding normal colonic mucosae by primer set 2 (Fig. 1). As shown in Fig. 2, the DNA of the tumor transplanted into nude mice from KUPL40 hybridized only with the mutated probe, and the DNA of the primary carcinoma from KUPL40 hybridized with both the mutated and the normal probes because of contamination of the normal mucosa. On the other hand, normal colonic mucosae from KUPL40 and other specimens hybridized only with a normal probe. These observations suggest that the mutation at codon 215 occurred only in the carcinoma from KUPL40 at the somatic level.

**p53 Gene Mutations in an Adenoma from a Patient with FPC.** The amplified region with primer set 2 where there was a mutation in the carcinoma from KUPL40 contained exons 5 and 6. This region is one of the hot spots in the human genome. Mutations of the p53 gene were frequently detected (17). To screen for other mutations in the amplified region with primer set 2, the 402-base pair PCR products from 78 tumors were digested with several restriction enzymes that recognize four base pairs. When PCR products were cleaved with HhaI and separated by polyacrylamide gel electrophoresis, adenoma from KUPL31 (KUPL31A) exhibited an abnormal band, in comparison with other specimens (data not shown). The PCR product generated from KUPL31A by primer set 5 was then cloned as described in “Materials and Methods.” We sequenced 4 independent clones and found 13-base pair deletions in exon 5 from codon 152 (CCG) to codon 156 (CGC) in 2 clones of 4, and the other clones were identical with the normal sequence from nonneoplastic cells (Fig. 3). Restriction fragment length polymorphism analysis with the p53 cDNA probe revealed that KUPL31A had allelic deletions in the p53 gene. The analysis was performed using tissues from 75 primary tumors, and the results are summarized in Table 1. There were 5 carcinomas from FPC patients with allelic deletions of the p53 gene. The carcinoma...
Fig. 2. Demonstration of p53 gene mutation in carcinoma from FPC patient KUPL40 by dot blot hybridization. Pn and Pm, oligonucleotides specific for normal and mutated sequences, respectively. Bold underlines, sequences corresponding to codon 215. PCR products generated with primer set 2 were hybridized with Pn (a) and Pm (b). Left to right, KUPL40N, A2, A3, C, C*, KUPL31N, P, C, and KUCO15N. C. KUPL, patients with FPC; KUCO, patients with NPCC. N, normal colonic mucosa; A, colorectal adenoma; C, colorectal carcinoma; C*, carcinoma transplanted into nude mice.

Table 1 Loss of heterozygosity for the p53 gene in colorectal tumors

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Normal Tissues Heterozygous</th>
<th>Loss of Heterozygosity</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPC</td>
<td>3/22 (14%)</td>
<td>5/9 (56%)</td>
</tr>
<tr>
<td>Adenoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carcinoma</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage.

from KUPL40 (KUPL40C) showed an allelic loss with D17S5, and the p53 cDNA probe was a noninformative case.

Mutations Detected by Denaturing Gradient Gel Electrophoresis. To screen for subtle alterations in the p53 gene, which could not be detected by restriction endonuclease mapping, we used the method of denaturing gradient gel electrophoresis (23). The 402-base pair PCR products, including 5 carcinomas with allelic deletion of the p53 gene from FPC, a carcinoma transplanted into nude mice from KUPL40 (KUPL40C*), and KUPL31A, were applied in the gradient gel at 60°C for 12 h. After electrophoresis, the gels were stained with ethidium bromide. KUPL40C* and KUPL31A with mutations in this region exhibited abnormal bands, but the others exhibited only a normal band. To confirm that these abnormal bands derived from neoplastic cells, PCR products from the corresponding normal colonic mucosa were also applied to the gradient gel (Fig. 4, a and b). KUPL40C* exhibited only the abnormal band, the position of which was lower than the normal band on the gel. On the other hand, KUPL31A exhibited two bands, one of which was abnormal with a higher position in the gel. In the case of other carcinomas, no abnormal band appeared. To confirm that these other carcinomas contained no mutation in the amplified region, we chose 5 of them and examined them by direct sequencing (31) to determine whether they had mutations in the amplified region. Single-stranded DNA generated by an asymmetric PCR using primers P< (0.5 pmol) and P6 (50 pmol), in which genomic DNA (1 μg) was used in a 50-μl PCR reaction for 30 cycles (96°C for 1 min, 60°C for 30s, and 72°C for 1 min), was sequenced using [γ-32P]ATP end-labeled primer P< as a sequencing primer. Thus by using direct sequencing, we obtained no evidence of mutations in these 5 cases, but we did find mutations in KUPL40C* and KUPL31A. And to determine whether the presence of nonneoplastic cells in tumor specimens makes it difficult to observe mutations by this method, we examined mutation p53 with varying mixtures of the DNA from normal cells and neoplastic cells (Fig. 4a). Fig. 4a shows four bands consisting of normal homoduplex, mutant homoduplex, and two different heteroduplex DNA fragments (32). The mutation in KUPL40 carcinoma is an A to G change, causing the domain to melt at a higher temperature, and therefore leads to a lower position in the gel. Two bands higher than the normal band in the gel are different heteroduplexes, each containing a single base mismatch. While we could detect the mutation in Fig. 4, Lane 1, in which the DNA ratio between normal colon mucosa and carcinoma transplanted into nude mice is 5:1, we could barely detect the mutation in Fig. 4, Lane 2 and 3, in which the DNA ratios are 10:1 and 50:1, respectively. Therefore, while some admixture with normal cells would not interfere with our ability to detect mutations, the level of sensitivity achieved with this technique remains to be defined.

Screening for Mutations by the Combined Methods of PCR and Denaturing Gradient Gel Electrophoresis. The PCR products generated by primer sets 2, 3, and 4 (Fig. 1) covered the four hot spots (17). When the 402-base pair PCR product generated by primer set 2 from 45 adenomas, 15 carcinomas from FPC, and 15 carcinomas from NPCC was applied in the denaturing gel with those from the corresponding normal co-
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![Diagram](image)

(a) KUPL40  
(b) KUPL31  
(c) KUPL36A

Table 2 Mutations in the p53 gene in colorectal tumors detected by denaturing gradient gel electrophoresis

<table>
<thead>
<tr>
<th>Tumor</th>
<th>No. of 17p alleles</th>
<th>Mutations in exon 5 or 6 or intron 5</th>
<th>Mutations in exon 7 or intron 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenoma</td>
<td>KUPL31A</td>
<td>1</td>
<td>+ (13-base pair deletion)†</td>
</tr>
<tr>
<td></td>
<td>KUPL36A5</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>KUPL50A</td>
<td>NA</td>
<td>+</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>KUPL40C</td>
<td>1</td>
<td>+ (ThrSer→Gly)</td>
</tr>
<tr>
<td>NPCC</td>
<td>KUPL31</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>KUPL36A5</td>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>KUPL50A</td>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

† The number of 17p alleles was determined by restriction fragment length polymorphism analysis, using D17S5 or p53 cDNA as a probe.

‡ Mutations were detected by denaturing gradient gel electrophoresis. +, existence of mutations.

‡‡ Information in parentheses, mutation in the p53 gene, by sequencing.

‡§ NI, noninformative case. For explanation of tumor designations, see legend to Fig. 2.

DISCUSSION

Previous studies have demonstrated that mutations in the p53 gene occurred in sporadic colorectal carcinomas from patients with NPCC. We attempted to determine whether mutations in the p53 gene also occur in tumors from FPC patients and in what stage of tumorigenesis the mutations occur. As shown in Table 2, we found p53 gene mutations in 3 adenomas and one adenocarcinoma from FPC patients and in 3 adenocarcinomas from patients with NPCC. We observed that all but 2 of these tumors with mutations had allelic losses of chromosome 17p. The results support the notion that the p53 gene is a recessive tumor suppressor gene, based on the theory that inactivations of the genes on both alleles is a hallmark of recessive tumor suppressor gene (10). However, there were several cases of allelic losses in the p53 gene in which we observed no mutation in the remainder of the p53 gene. The rate of loss of heterozygosity on the p53 gene was 14% (3 of 22) in colorectal adenomas and 56% (5 of 9) in carcinomas from FPC patients and 43% (3 of 7) in carcinomas from NPCC cases. We found no mutation in 5 carcinomas with an allelic loss of the p53 gene from FPC. Factors worthy of consideration include: (a) mutations capable of affecting the transcription of p53 mRNA, its stability, or its translational capacity (17) exist in domains melting at a higher temperature reach final gel positions indistinguishable from the wild type (23); (d) contaminations of nonneoplastic cells made it difficult to detect mutations, because these contaminations resulted in abnormal bands of low intensity in the case of some admixture with normal cells (Fig. 4); and (e) only an allele loss in the p53 gene may lead to progressive disorder of controlling growth. Once one of the p53 genes is deleted, progression to malignancy may occur in cells from FPC patients with inactivation of the FPC major gene on chromosome 5q (11, 12). This hypothesis could explain previous observations that loss of heterozygosity on chromosome 17p and that of chromosome 5q did not occur simultaneously in tumors from FPC, although losses on both chromosomes 5 and 17 were frequently observed in the case of NPCC (6).

We found mutations in 3 adenomas of 45 cases of FPC. Two (KUPL31A, 36A) of the 3 cases had allelic deletion in the p53 gene, and the other (KUPL50A) was not an informative case. Polyps from KUPL31 were about 0.5 cm in diameter, the pathology being a tubular adenoma. Polyps from KUPL36 were 0.2–1.0 cm in diameter, and the pathology was tubular and tubulovillous adenoma. These results show that genetic changes in the p53 gene occur in benign adenomas. In view of the
multistep theory of carcinogenesis, it is probable that adenomas will have undergone several gene or chromosomal mutations before revealing the fully malignant state. Mutations of the p53 gene may play an important role in forming adenomas or in the progression from adenoma to adenocarcinoma. The p53 protein has been well conserved during evolution, and regions of the p53 gene with mutations coincided well with the most highly conserved region of the gene (33).

These results suggest that p53 may play an important role in tumorigenesis both in adenomas and in carcinomas from FPC patients as well as in sporadic cases. In cases of colorectal tumorigenesis in FPC, several genetic alterations may be involved, in addition to the mutated FPC gene, including activation of ras genes (1-3), mutations in the p53 gene, etc., and some of these genetic changes may interact with one another.

Denaturing gradient gel electrophoresis has been used to detect mutations and polymorphisms in genes (23, 34). As for the combined method of PCR and denaturing gradient gel electrophoresis, an oligomer with a G-C clamp was found to be effective in separating the different fragments (34). We used simple oligomers without G-C clamps and separated the abnormal fragments from normal ones, although the level of sensitivity achieved with this technique remains to be defined. Further investigations of the G-C clamp method will be required to estimate the true frequency of mutations. PCR combined with denaturing gradient gel electrophoresis may be one of the easiest methods, without the use of radioisotopes, of screening for mutations in identified as well as presumptive tumor suppressor genes.

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