Mutations of the p53 Gene in the Stool of Patients with Resectable Colorectal Cancer

Susumu Eguchi, M.D.
Norihiro Kohara, M.D.
Koh Komuta, M.D.
Takashi Kanematsu, M.D.

Department of Surgery II, Nagasaki University School of Medicine, Nagasaki, Japan.

Presented at the Seventh Annual Meeting of the Japanese Research Society for Gastroenterological Carcinogenesis, Oita, Japan, September 8–9, 1995.

The authors thank Professor H. Shiku and M. Ohara for their helpful comments.

Address for reprints: Takashi Kanematsu, M.D., Department of Surgery II, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852, Japan.

Received December 4, 1995, accepted December 8, 1995.

BACKGROUND. This study was undertaken to evaluate whether genetic analysis in the stool can be useful for detecting malignant tumors in the colon and rectum. We searched for the possible presence of mutations in the p53 gene in the stool of patients with resectable colorectal cancer. Alterations in the p53 gene are the most frequent among mutant genes related to colorectal cancer.

METHODS. Surgically resected tumor specimens and stool samples from 25 patients with colorectal cancer were examined for mutations of exons 5–8 of the p53 gene by polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP). Results were compared with those achieved by fecal occult blood testing.

RESULTS. Mutations of the p53 gene were found in the tumor tissues in 11 of 25 patients (44%). Of these 11 patients, 7 (64%) had evidence of alterations in the p53 gene within the stool. Of five patients who were negative for fecal occult blood testing, p53 mutations in the stool were evident in three patients.

CONCLUSIONS. This method of stool DNA analysis for tumor-specific mutations is expected to have a wide application in clinical screening for colorectal cancer. *Cancer* **1996; 77:1707–10.** © *1996 American Cancer Society.*

KEYWORDS: p53 gene, DNA analysis, stool, colorectal cancer.

Previously, p53 gene mutations have been identified in the urine samples of patients with a high proportion of primary urinary bladder cancer,[1] and *ras* oncogene mutations have been detected in DNA purified from the stool of nine patients with curable colorectal tumors.[2] Alterations in the p53 gene are the most frequent among mutant genes related to colorectal cancer and mutations have been observed in 50–86% of colorectal cancers.[3–6]

We searched for the possible presence of the same mutations in the p53 gene in resected colorectal specimens and stool samples using the polymerase chain reaction single-strand conformation polymorphism analysis,[7] with the objective of establishing a noninvasive and sensitive screening method for colorectal cancer.

MATERIALS AND METHODS

Sixteen Japanese men and 9 women (average age, 64 years) with histologically proven carcinoma of the colon and rectum were studied. One patient (Patient 8, Table 1) had a concomitant adenoma. Prior to surgery, the colon was cleansed with a laxative and kanamycin and metronidazole were given for 2 days. Stool samples were collected one day prior to surgery. Tumor samples were obtained from the surgically resected specimens. All samples were stored at a temperature of −80 °C.

DNA Preparation

DNA was first purified from the stool and tumor tissues using Sepagene® (Sanko Junyaku Co. Ltd., Tokyo, Japan). Briefly, approximately 100 mg

TABLE 1
Clinical Characteristics

			Tumor					DNA (μg)[g]		Mutant p53[h]	
Patient	Age	Sex	Location[a]	Size (cm)	Dukes'[b]	Histology[c]	F.O.B.[d]	Tumor	Stool	Tumor	Stool
1	76	M	Tr	1.4 × 0.9	A	Pap.	–	283	28		
2	79	M	R	6.3 × 7.5	—	Mod.	+	176	×[f]		
3	73	M	Tr	4.7 × 6.2	B	Well	+	365	400 (PCR×)[e]		
4	81	F	R	2.0 × 1.5	A	Mod.	+	72	974		
5	40	M	As	4.5 × 4.5	C	Mod.	+	66	×[f]		
6	73	M	Rs	2.8 × 2.2	C	Mod.	+	154	200	+ (Exon 5)	–
7	71	M	R	2.4 × 2.3	A	Mod.	+	144	22		
8	52	M	R	12 × 5.0	C	Mod.	+	530	324	+ (Exon 7)	+
				7.5 × 3.5	—	Adenoma		340			
9	66	M	Tr	4.0 × 2.8	B	Well	–	126	98		
10	67	M	R	8.0 × 6.5	B	Mod.	+	62	246		
11	45	F	R	2.8 × 2.1	C	Mod.	–	332	410	+ (Exon 7)	+
12	68	F	R	10.0 × 4.0	C	Poor	+	120	146		
13	65	F	R	9.0 × 7.0	B	Mod.	+	60	178	+ (Exon 5)	–
14	56	F	Total	F.P.[i]	C	Mod.	+	2900	164	+ (Exon 8)	–
15	62	M	R	7.0 × 5.0	C	Mod.	+	1418	152		
16	69	F	SIg	6.0 × 3.9	C	Mod.	+	1900	108		
17	72	F	SIg	3.0 × 2.8	C	Mod.	–	526	88	+ (Exon 6)	+
18	65	F	R	5.0 × 3.5	C	Mod.	+	780	800	+ (Exon 6)	–
19	51	M	SIg	4.0 × 4.0	B	Well	+	342	50		
20	65	M	R	6.0 × 5.2	C	Mod.	+	748	4700	+ (Exon 7)	+
21	51	M	SIg	3.7 × 3.5	C	Mod.	+	184	16	+ (Exon 6)	+
22	72	M	P	6.5 × 4.2	C	Mod.	+	88	228		
23	50	M	R	5.3 × 4.3	B	Mod.	–	984	50	+ (Exon 7)	+
24	68	F	P	6.3 × 5.5	B	Well	+	304	1536		
25	58	M	R	7.5 × 3.1	C	Mod.	+	258	110	+ (Exon 5)	+

[a] As.: ascending colon; Tr.: transverse colon; Sig.: sigmoid colon; R.: rectum; P.: anal canal; Total: a case of familial polyposis. Numerous polyps were present all over the colon.
[b] Dukes' classification.
[c] Histological type. Pap.: papillary adenocarcinoma; Well: well differentiated adenocarcinoma; Mod.: moderately differentiated adenocarcinoma; Poor: poorly differentiated adenocarcinoma.
[d] F.O.B.: Fecal occult testing.
[e] DNA was extractable but not amplifiable.
[f] DNAs were not extractable.
[g] Concentration of the DNAs at 260 nm.
[h] Detection by polymerase chain reaction-single-strand conformation polymorphism analysis of exons 5, 6, 7, and 8 of the p53 gene.
[i] F.P.: a case of familial polyposis with evidence of malignancy.

of the stool frozen at −80 °C was diluted with 100 μL of Tris HCl. Proteins were digested with 100 μL of guanidine thiocyanate and extracted with 700 μL of bentonite chloroform and 400 μL of sodium acetate without phenol. After ethanol precipitation, the DNAs were dried using a decompression drier and diluted with Tris-ethylenediamine tetraacetic acid (EDTA). The concentrations of DNAs were measured at 260 nm using a spectrophotometer, and are listed in Table 1.

Polymerase Chain Reaction

Exons 5–8 of p53 gene were then amplified from each DNA by polymerase chain reaction (PCR), and subjected to polyacrylamide gel electrophoresis. Oligonucleotide primers for PCR amplification were synthesized in a 380B DNA synthesizer. The following four sets of primers, 5U-5D, 6U-6D, 7U-7D, and 8U-8D, were used to amplify exons 5, 6, 7, and 8, respectively, of the p53 gene. Sense primers used were: 5U, 5′-GGGGATCCTCTTCCTGCAGTACTC-3′; 6U, 5′-GTCTGGCCCCTCCTCAGCAT-3′; 7U, 5′-GGGGATCCTAGGTTGGCTCTGACT-3′; and 8U, 5′-GGGGATCCTATCCTGAGTAGTGGT-3′ (each primer contained a benzamide HI site). The antisense primers were: 5D, 5′-GCAAATTTCCTTCCACTCGG-3; 6D, 5′-GGAATTTGCAAACCAGACCTC-3′; 7D, 5′-GGGAATTCAAGTGGCTCCTGACCT-3′; and 8D, 5′-GTCCTGCTTGCTTACCTCG-3′ (each primer contained an Eco RI site). PCR was performed using a thermal cycler (PC-70, Astec, Fukuoka, Japan) with 2 μL of genomic DNA in a total volume of 20 μL containing 8 μL of [alpha-p^{32}]γATP. Thirty-nine cycles

comprised of 90 seconds at 92 °C for denaturation, 90 seconds at 55 °C for annealing, and 1 minute at 72 °C for extension were run.

Single-Strand Conformation Polymorphism

The PCR products were examined by Single-Strand Conformation Polymorphism analysis to identify mutations in the p53 gene. The PCR mixture was heated at 90 °C with 7 μL of a formamide dye mixture (95% formamide, 20 mM EDTA, 0.05% xylene cyanol, and 0.05% bromphenol blue), and 1.5 μL of the mixture was applied to a 6% polyacrylamide gel containing 50 mM Tris-borate. Glycerol (5%) was also added. Electrophoresis was performed at 1800 volts with cooling by a fan for 2 hours at 4 °C and for 1.5 hours at room temperature. The gel was dried on filter paper and exposed to X-ray film for 24 hours at room temperature. Polymerase chain reaction-single-strand conformation polymorphism analysis was performed in duplicate.

Fecal Occult Blood Testing

To search for occult blood in the stool, the gold colloid immunochromato method which reacts on human hemoglobin, was used.[8]

RESULTS

DNA was extracted from all tumor tissues, but in two patients (Patients 2 and 5), DNA could not be extracted from the stool (Table 1). Mean concentrations of extracted DNA in the tumor were 510 ± 558 μg/mL (range, 60–2900 μg/mL), and 561 ± 1168 μg/mL (range, 16–4 700 μg/mL) in the stool. Amplification of exons 5–8 was successful in the tumor specimens of all patients, whereas it was unsuccessful in the stool sample in one patient (Patient 3; Table 1).

Mutations of the p53 gene were detected in exons 5–8 in the tumors of 11 of 25 patients (44%). Mutations were evident in exons 5 and 6 in three patients each, exon 7 in four patients, and exon 8 in one patient. Among the 11 patients, mutations of the p53 gene were identified in the stool of 7 patients (64%) (Fig. 1). However, there were no patients who showed the presence of mutations in the p53 gene in the stool of the abscence of the mutation in the tissue (Table 1). No significant difference in clinicopathologic variables, including location and size of tumors and stage of the disease, was noted between groups with or without evidence of the p53 gene mutations in the tumor or stool samples.

Among the 25 patients, occult blood was positive in the stool of 20 patients (80%). In the remaining five patients who were negative for occult blood, mutations of the p53 gene were identified in three (Patients 11, 17, and 23, Table 1).

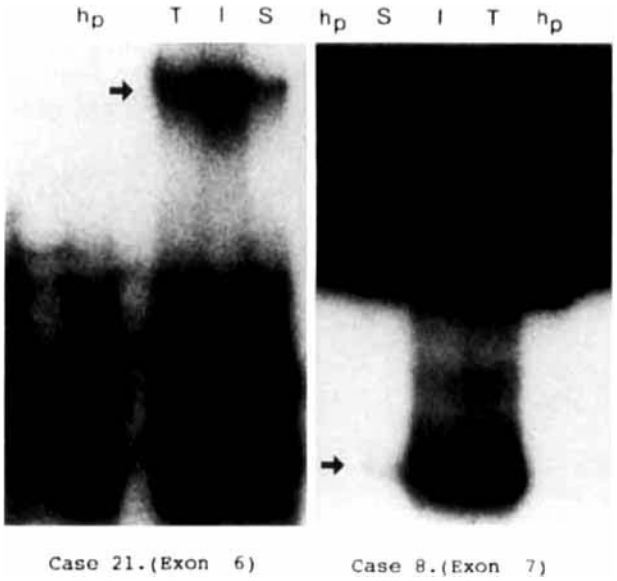

FIGURE 1. Detection of p53 mutations by polymerase chain reaction single-strand conformation polymorphism analysis. hp represents normal human placenta DNA. Arrowheads indicate bands that migrated at different positions from the normal ones. DNA from the stool sample was the same migration as DNA from the tumor in 7 of 11 patients (e.g., 8, Patient 21). T: tumor with mutant p53 genes; S: stool from the patient whose tumor was with mutant p53 genes.

DISCUSSION

There were 156,000 new cases of colorectal cancer diagnosed annually in the United States in 1992, and 58,200 patients died of the malignant disease,[8] although a slow decline in the mortality of patients with this disease was reported.[9] Early detection of colorectal cancer favorably alters the clinical course. Fecal occult blood testing has been the technique used to detect neoplasms in the large bowel. However, the measurement of occult blood in the stool using immunochemical methods is unsatisfactory because many tumors do not bleed.[11]

A common cancer-related gene change that can occur at the gene level in the colon is a p53 mutation,[3,4] and nearly all existing tumors can be detected by analysis of the four mutational hot spots, exons 5, 6, 7, and 8.[11,12] In the present study, the p53 mutations were detected in the tumor tissues of 11 of 25 patients (44%); among these 11 patients, genetic alterations in the stool were found in 7 patients (64%). In addition, p53 mutations were detected in the stool of three patients with negative fecal occult blood testing.

Thus, this genetic approach can be useful for detecting a malignant tumor in the colon and rectum. Moreover, it also may be useful for detecting colorectal cancer in patients with false-negative fecal occult blood testing. However, although the present study illustrates the prin-

ciple that specific gene changes can be detected in the stool, molecular genetic approaches are not readily adaptable for screening for colorectal cancer. These analyses should provide criteria for a screening test because the simplicity, economic, feasibility, sensitivity, noninvasiveness, and overall accuracy of molecular genetic approaches are substantially better than those of fecal occult blood testing.[13] Nevertheless, the results show that this strategy may serve as a pertinent method for screening individuals who are at high risk for colorectal cancer, particularly those with inherited syndromes, a personal history of adenoma or colorectal cancer, and inflammatory bowel disease.

REFERENCES

1. Sidransky D, Eschenbach DV, Tsai YC, Jones P, Summerhayes I, Marshall F. Identification of p53 gene mutations in bladder cancers and urine samples. *Science* 1991;252:706–9.
2. Sidransky D, Tokino T, Hamilton SR, Kimzler KW, Levin B, Fost P. Identification of ras oncogene mutations in the stool of patients with curative tumors. *Science* 1992;256:102–5.
3. Vogelstein B, Feron ER, Hamilton SR, Kerns E, Preisinger AC, Leppert M. Genetic alterations during colorectal tumor development. *N Engl J Med* 1988;319:525–32.
4. Baker SJ, Presinger AC, Jessup JM, Paraskeua C, Markowitz S, Willson JKV. p53 gene mutations occur in combination with 17p allelic detection as late events in colorectal tumorigenesis. *Cancer Res* 1990;50:7717–22.
5. Editorials. Screening for colorectal cancer by stool DNA analysis. *Lancet* 1992;339:1141–2.
6. Bertek J, Bartkova J, Vojtesek B, Staskoua Z, Lukas J, Rejthar A. Aberrant expression of the p53 oncoprotein is a common feature of a wide spectrum human malignancys. *Oncogene* 1991;6:1699–703.
7. Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc Natl Acad Sci USA* 1989;86:2766–70.
8. Boring CC, Squires TS, Tong T. Cancer statistics. *CA Cancer J Clin* 1992;42:19–38.
9. Levin B. Colorectal cancer screening. *Cancer* 1993;72:1056–60.
10. Ahlquist DA. Occult blood screening. *Cancer* 1992;70:1259–65.
11. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. *Science* 1991;253:49–53.
12. Nigro JM, Baker SJ, Preisinger AC, Jessup JM, Hostetter R, Cleary K. Mutations in the p53 gene occur in diverse human tumour types. *Nature* 1989;342:705–8.
13. Caffarey SM, Broughton CIM, Marks CG. Faecal occult blood screening for colorectal neoplasia in a targeted high-risk population. *Br J Surg* 1993;80:1399–400.