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p53 and Follow-up of Colorectal Adenocarcinomas

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Circulating p53 antibodies (ELISA method), p53 genetic alterations (SSCP), and protein overexpression (immunohistochemistry) were studied in 41 patients with colorectal adenocarcinomas and 10 control patients. Carcinoembryonic antigen (CEA) and carbohydrate antigen 19.9 (CA 19-9) were evaluated in parallel. Ten patients with p53 antibodies and p53 overexpression were selected. Tumor DNA extracts from these 10 patients were analyzed by SSCP. Of all 41 patients, 10 (24%) showed significant levels of p53 antibodies, and p53 accumulation was detected in 20 (48%) patients. In six patients, p53 antibodie concentrations decreased rapidly after surgery; in two patients, these levels returned to normal values. Of the 10 selected tumors, eight revealed TP53 gene mutations. Only two patients with high values of both CEA and CA 19-9 developed p53 antibodies. In conclusion, beside classical tumor markers, circulating p53 antibodies may be considered as additional markers for the management of patients with colorectal adenocarcinomas.

KEY WORDS: p53; p53 antibodies; colorectal adenocarcinoma; TP53 gene mutations.

The TP53 gene has been the subject of intense investigation since it was recognized that mutation in this tumor suppressor gene was the most frequent genetic alteration in human cancers (1–3). The most common alteration is mutation of one allele (usually a missense point mutation) and deletion of the other (1). TP53 gene mutations occur in 50–60% of colorectal adenocarcinomas (4).

p53 alterations in colorectal cancer have been studied using three main approaches: (1) Molecular analysis of the TP53 gene has been done by PCR amplification and DNA sequencing on tumor specimens (5). (2) Immunohistochemical analysis of tumor tissues has been accomplished based on the fact that missense mutations result in conformational changes that stabilize the protein. The mutated p53 protein has a longer half-life than wild-type p53 protein and is overexpressed in the nucleus of tumor cells (6). Some mutant p53 proteins are able to complex with the heat-shock protein (hsp 70) (7). (3) Detection of p53 antibodies in sera of cancer patients has been the object of a variety of studies conducted to clarify the mechanisms of p53 antibodies production in cancer patients (8–10). However, the humoral immune response against the p53 mutant protein is not completely understood. Numerous studies have confirmed that the presence of circulating p53 antibodies is a consequence of p53 overexpression (11, 12). The accumulated mutant p53 protein may be considered as an immunogen and induce p53 antibodies production. It was also shown that p53 antibodies are produced against wild-type and mutant p53 protein (10).

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In the present study, we looked for the presence of serum p53 antibodies in patients with colorectal adenocarcinomas. Relationships between serum levels of p53 antibodies and p53 genetic alterations and overexpression were investigated in order to provide more information about diagnosis and monitoring of patients with colorectal cancer.

MATERIALS AND METHODS

Patients

Forty-one patients (21 women and 20 men) with adenocarcinoma of colon (29 patients) or rectum (12 patients), treated by surgical resection at Nimes Hospital between June 1995 and October 1996 were included in this study (Table 1). The median age was 71 years (range 45–89). All patients with colon carcinoma had undergone colic resection. Three of 12 patients with rectal carcinoma received preoperative irradiation.

Tumor Tissue

According to the Dukes' staging system, 2 patients were stage A, 2 stage B1, 18 stage B2, 8 stage C1, 5 stage C2 and 6 stage D. The site of the primary tumor was the rectum in 13 cases, the left colon in 15 cases, and the right colon in 13 cases. According to histology, adenocarcinomas were classified as poorly differentiated (2 cases), moderately differentiated (22 cases), well differentiated (14 cases), and mucinous carcinomas (3 cases). Immediately after surgery, some tumor material for biological studies was stored at −80°C for p53 gene mutation analysis and some was processed for immunohistochemical p53 expression.

Serum Samples

Serum samples of the 41 patients with colorectal cancer were collected before surgery and then 1, 3, 6, 12, and up to 16 months after resection of the primary tumor. Serum samples of 10 control patients (20–25 years) without digestive diseases were also collected. Samples were stored at −20°C before analysis.

Assay

The detection of p53 antibodies in patient sera was performed with a commercially available sandwich enzyme-linked immunosorbent assay (Dianova, Hamburg, Germany). This assay has been validated by comparing data with those obtained by western blotting with recombinant p53. The 379 noncancer patients had negative p53 antibody assay results (13). This assay was used for semiquantitative detection of circulating p53 antibodies. In order to improve the follow-up of patients, we have developed a quantitative assay with a calibration curve. We used a mouse anti-human p53 monoclonal antibody (clone PAB 122; Boehringer Mannheim Biochemica) and a goat antibody, anti-mouse IgG, conjugated with peroxidase (Anti-mouse IgG; Immunotech).

Table 1. Clinical Characteristics of 41 Patients with Colorectal Adenocarcinomas

Patients	*Sex*	*Age (yr)*	*Dukes' stage**	*Tumor site†*
1	M	72	D (lm)	L
2	F	79	D (lm)	R
3	F	76	C1	L
4	M	67	C2	L
5	M	74	D (lm)	R
6	M	78	B1	R
7	F	49	C1 (lm)	R
8	M	87	C2	R
9	M	77	C1	R
10	M	86	B2	L
11	M	66	C1	L
12	F	70	D (lm + bom + brm)	L
13	F	58	B2	Rectum
14	F	89	C1 (skm)	R
15	F	69	B2	R
16	F	74	B2	L
17	F	83	B2	R
18	F	69	C2	Rectum
19	M	68	B2	Rectum
20	M	79	B2	L
21	M	66	B2	R
22	M	71	B2	R
23	M	71	A	Rectum
24	M	73	B2	L
25	F	61	C1	Rectum
26	F	61	C1	Rectum
27	M	62	B2	L
28	F	45	A	Rectum
29	M	68	C2 (lm)	Rectum
30	M	63	D (lm)	R
31	F	82	B2 (ot)	L
32	M	72	B2	L
33	F	70	B2	Rectum
34	F	74	C2	L
35	F	48	B2	L
36	F	65	C1	Rectum
37	M	78	B2	L
38	F	72	D (pc)	Rectum
39	F	70	B2	Rectum
40	M	81	B2	Rectum
41	F	73	B1	R

* lm: liver metastasis; bom: bone metastasis; brm: brain metastasis; skm: skin metastasis ot: ovarian tumor; pc: peritoneal carcinosis.
† L: left colon; R: right colon.

Carcinoembryonic Antigen and Carbohydrate Antigen 19.9

CEA and CA 19-9 assays were performed using radioimmunoassay kits. The Behring kit was used for CEA and the Cis Bio International kit for CA 19-9. Cut off values were respectively 10 IU/liter and 35 IU/liter.

Immunohistochemistry (p53 Overexpression)

Paraffin sections were cut at 5 μm, mounted on slides, dewaxed for 10 min in toluene, and rehydrated through graded alcohols and washed in water. A preliminary treatment was performed by incubating deparaffinized slides for 5 min twice in citrate buffer, in a microwave oven. Sections were then left at room temperature for 20 min. They were

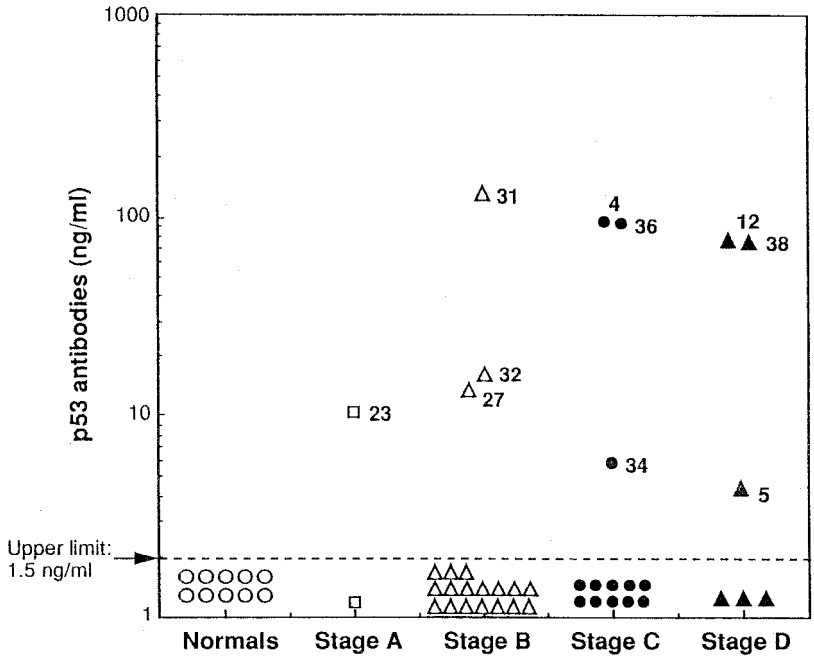

Fig 1. Serum p53 antibodies concentrations. Concentrations of p53 antibodies in serum of 10 healthy subjects and 41 patients with colorectal carcinomas were evaluated by ELISA as described in Materials and Methods. Values are reported as determined on a standard curve with monoclonal p53 antibodies as standard. Numbers indicated on the graph are those of patients reported on Table 1.

rinsed with phosphate-buffered saline (PBS), and endogenous peroxidase was removed by soaking in 3% hydrogen peroxyde for 5 min. Sections were rinsed in PBS before incubation with the monoclonal antibody DO-7 for 30 min (antibody DO-7 recognizes a fixation resistant epitope at the N terminus of the human p53 protein and reacts with both wild-type and mutant proteins). Using aminoethyl carbazole (AEC) as substrate, the avidin–biotin peroxidase method was carried out according to the manufacturer's recommendations. Slides were counterstained with hematoxylin and mounted with an aqueous mounting medium. Normal rectum and colon sections, used as internal negative controls, demonstrated no immunoreactivity. Tumors with strong p53 overexpression were used as positive controls.

p53 Gene Mutation Analysis

DNA Extraction. Genomic DNA was prepared from frozen tissue by grinding the sample to a powder in liquid nitrogen, using a mortar. The powder was then dissolved in an extraction buffer containing PBS, pH 7.4. DNA extraction was performed with a commercial extraction kit (genomic DNA Isolation Kit, Clontech). The extracted DNA was frozen at −30°C in Tris-EDTA buffer (10 mM Tris-HCl/1 mM EDTA, pH 7.4).

Amplification by Polymerase Chain Reaction. The extracted DNA was diluted to a final concentration of 4–6 ng/μl. Exons 2–11 of the p53 gene were amplified separately, using oligonucleotides (Human p53 Amplimer Panels, Clontech). The reaction mixture (50 μl) contained 300–500 ng DNA, 2 units of Taq polymerase (Promega), Taq polymerase buffer, pH 9, 1.5 mM $MgCl_2$, 20 pmol of each primer, and 0.2 mM dNTP (Promega). The 35 amplification cycles were carried out in a DNA minicycler (PTC-150 model, version 1.2, MJ Research). Cycles were programmed as follows: 30 sec at 95°C (denaturation), followed by 45 sec at 66°C (annealing), and 1.30 min at 72°C (extension). A final extension for 7 min completed the cycling reactions. Amplified products were cooled at +4°C, then stored at −30°C.

SSCP Analysis. PCR products (20 μl) were denatured in 10 μl of methylmercuryhydroxide (MMH) loading buffer [Tris, boric acid, EDTA (TBE) containing 20 mM MMH, 0.05% bromophenol blue, 0.05% xylene cyanol and glycerol 1%] by boiling for 5 min and then kept on ice until loading. The mixture (30 μl) was submitted to electrophoresis on a nondenaturing 4–20% polyacrylamide gel gradient (49:1 acrylamide–bisacrylamide, 800 mm high × 1 mm thick). Electrophoresis was carried out at 4°C, with TBE 1× (300 V for 1 hr) using a vertical electrophoretic chamber (Hoefer Scientific Instruments, San Francisco, California). Gels were stained with ethidium bromide. Samples displaying mutant bands were reamplified and run a second time to confirm the existence of one or more mutations.

RESULTS

Circulating p53 Antibodies

Figure 1 reports p53 antibody values obtained in

TABLE 2. BIOLOGICAL PARAMETERS OF 41 PATIENTS WITH COLORECTAL ADENOCARCINOMAS *

Patient	Immuno-histo chemistry (%)	SSCP exon involved	Before surgery CEA	Before surgery CA 19-9	p53 antibodies (ng/ml) (2) Before surgery	After resection (months) 1	3	6	12	>12	Follow-up
1	0		2100	28000							
2	0		8.5	180							
3	<15		3.2	12							
4	**100**	**4 and 5**	**1.9**	**4**	**96.2**	**45.9**	**38.7**				9 months/good clinical conditions
5	**100**	**5 and 9**	**4.2**	**10**	**4.5**						Died at 6 months
6	15		4.7	30							
7	75		1.4	37							
8	15		3	29							
9	50		14	3.5							
10	<15										
11	<15										
12	**100**	**7**	**19**	**99**	**77.2**	**61.6**	**89.4**	**91.8**	**29.2**	**17.2**	Died at 17 months
13	80		0.6	1							
14	0										
15	<15		1.1	4							
16	50		2	5							
17	<15		3.8	6							
18	<15		135	60							
19	<15		2	38							
20	80		12	66							
21	<15		1	4.5							
22	<15		400	330							
23	**100**	**4 and 5**	**1.2**	**3**	**10.5**	**7.2**		**5.4**			12 months/good clinical conditions
24	0		8	12							
25	100		1.3	4							
26	80		4.5	28							
27	**80**	**7**	**3.9**	**9**	**14.7**	**1**					Died at 1 month
28	<15		4	12							
29	80		28	155							
30	<15		1	45							
31	**80**		**1.5**	**18**	**131.4**	**131.1**	**129.5**	**145**	**144.2**		18 months/stable clinical conditions
32	75	**5**	**0.3**	**9.1**	**16.7**	**9.8**				**0.5**	12 months/good clinical conditions
33	<15		3.6	12							
34	**85**	**4, 5 and 9**	**3**	**30**	**6.1**						9 months/good clinical conditions
35	<15		1	4							
36	**80**	**4**	**84**	**4400**	**92.8**	**95.8**					10 months/good clinical conditions
37	0		3.5	10.5							
38	**50**		**0.8**	**1**	**76.9**	**52.1**	**51.3**	**72**			15 months/bad clinical conditions
39	<15		1	40							
40	50		1.1	12.6							
41	80		3.7	13							

* Values for immunohistochemistry represent percentage of tumor cells labeled with anti-p53 antibodies (DO-7). Values for CEA and for CA 19-9 are expressed as IU/liter (normals: <10 for CEA and <35 for CA 19-9). For p53 antibodies, the upper limit for normals was 1.5 ng/ml. Values in bold type represent patients positive for p53 antibodies.

sera of the 10 normals and 41 patients before surgery. Based on clinical criteria, upper value for noncancer patients was 1.5 ng/ml. In these conditions, 10 of 41 patients with colorectal cancer were positive. p53 antibodies were found in patients whatever the clinical stage of the disease. Serum p53 antibodies were tested before treatment and within the follow-up period. Results reported in Table 2 showed two different groups:

One group was patients without p53 antibodies [31/41 (76%)]. Among these 31 patients without p53 antibodies, 10 displayed p53 overexpression of >15%.

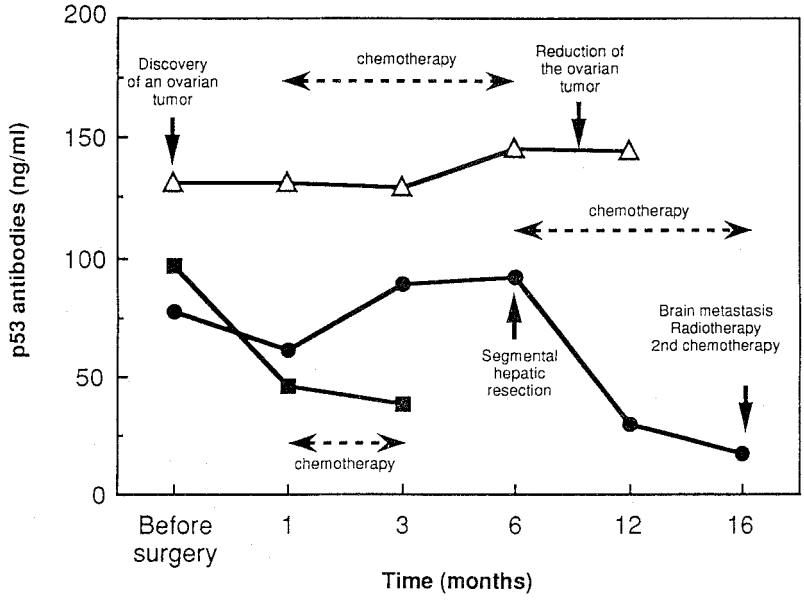

Fig 2. Follow-up of three typical patients. Serum concentrations of p53 antibodies were measured as indicated in Materials and Methods before and during months after surgery (patient 12: circles; patient 31: triangles; patient 4: squares). Events occurring during this follow-up are indicated on the graph.

Serological analysis during the follow-up was performed on 21 patients. None of the patients without p53 antibodies developed p53 antibodies during the follow-up. Patient 29 is described in detail: this 68-year-old male patient had undergone surgical resection for a rectal adenocarcinoma (Dukes' C2) in July 1995. Immunohistochemical analysis of the resected specimen showed p53 overexpression of >80%. p53 antibodies remained negative, although pulmonary and hepatic metastases were discovered. The patient died after six months. CA 19-9 and CEA values remained elevated during the follow-up.

The other group was patients with p53 antibodies [10/41 (24%)]. All 10 positive patients showed p53 overexpression of >50%, demonstrating the close correlation between the accumulation of the p53 protein in tumor cells and the presence of circulating p53 antibodies. Two of the 10 patients died during the 12-month follow-up. Patient 12 died at 17 months. The eight patients investigated may be subdivided into two subgroups:

Five patients had elevated p53 antibodies values during the follow-up. These patients received postoperative chemotherapy consisting of a combination of 5-fluorouracil and folinic acid (5-FU-FOL) except for patient 31, who received carboplatin. One month after tumor resection, p53 antibodies values were still high, and quite near the values before surgery. Patient 4 was the only one who showed a >50% drop in p53 antibody serum concentration one month after surgery. Ganglionic or visceral metastases were observed in this subgroup.

Three patients had decreased p53 antibodies values. Preoperative values of p53 antibodies were moderately high compared with the first subgroup and returned to normal values in two cases (patients 32 and 27) and remained above the normal range in the other case (patient 23). No ganglionic metastases was observed in this subgroup.

Follow-up of Three Typical Patients (Figure 2)

Patient 12. This 70-year-old woman was operated on in July 1995 for a Dukes' D adenocarcinoma of the left colon. Preoperatively, liver metastases were discovered but not resected. In January 1996, the patient underwent segmental hepatic resection and received, in March 1996, chemotherapy with 5-FU-FOL. In August 1996, brain metastases were discovered, and she received radiotherapy and a second course of chemotherapy. The patient died in December 1996. Serum concentrations of CEA and p53 antibodies were performed within the 16-month follow-up. Preoperative CEA values were above the normal limit and increased after the first operation. CEA concentration decreased after the second operation, but still remained elevated. A small decrease in p53 antibody

values was found after tumor resection. Three months after surgery, p53 antibodies values were higher than preoperative values. After segmental hepatic resection, concentrations of p53 antibodies decreased significantly but remained above the normal limit. Immunohistochemistry on the resected metastasis displayed p53 overexpression above 80%. This patient died at 17 months.

Patient 31. This 82-year-old woman had a Dukes' B2 adenocarcinoma that was diagnosed in November 1995. Preoperatively, an ovarian tumor was discovered but was inoperable. During six months, the patient received chemotherapy with carboplatin. At the ninth month, the ovarian tumor was reduced. At the end of the first year, colonoscopy did not identify any local tumor relapse and tomodensitometry confirmed the stability of the ovarian tumor. Serum concentrations of CA 19-9 and CEA were within the normal limit in the preoperative period and during the follow-up. CA 125 values were above the normal range and remained at the same level during the follow up. Before surgery, this patient had high values of p53 antibodies, and these values remained constant during 12 months. It is consistent with the fact that p53 antibody concentrations were relevant to the ovarian tumor. At 18 months' follow-up, this patient's clinical status may be considered as stable.

Patient 4. This 67-year-old man had a Dukes' C2 adenocarcinoma. p53 antibodies values were elevated before surgery and decreased significantly one month after resection of the tumor. The decrease in serum p53 antibody values was correlated with the clinical history. CEA and CA 19-9 values were normal before surgery. At nine months' follow-up, this patient was in good clinical condition.

p53 Overexpression

p53 protein overexpression was investigated using immunohistochemical analysis. The monoclonal antibody DO-7 was used to stain paraffin sections. Previous studies have demonstrated that DO-7 antibody was one of the most sensitive to detect p53 overexpression (14–18). In the present study, positivity was limited to nuclei of tumor cells. Sections with nuclear staining in 15% or less of the tumor cells were considered as negative. Overexpression was detected in 20 of 41 (48%) of the tumor specimens examined (Table 2). The level of p53 overexpression was similar for patients with or without p53 antibodies. The concordance of results between immunohistochemistry and p53 antibodies (ie, both positive and both negative) was 75% (31/41). Ten of these 20 patients (50%) with p53 overexpression had p53 antibodies in serum.

Patients 12 and 17 displayed p53 overexpression in liver metastases of 85% and 100%, respectively.

SSCP Analysis

SSCP analysis was performed on 10 tumor tissues from patients when p53 overexpression and p53 antibodies were detected (Figure 3). p53 exons 2–11 were analyzed. Mutant p53 was identified by the presence of one or two extra bands migrating above or below the control product. All mutations were confirmed by reamplifying the samples and running them on separate SSCP gels. Mutations were found in 8/10 adenocarcinomas. In three carcinomas, DNA mobility shifts were present in two exons: exons 4 and 5 were involved in two cases, exons 5 and 9 in one case. In another sample, three mutations were found in exons 4, 5, and 9. Two specimens failed to display any mutation by SSCP.

DISCUSSION

The major objective of the present work was to study p53 overexpression (immunohistochemistry) and mutations (SSCP analysis), in relation to outcome in patients who showed elevated values of p53 antibodies in serum. To our knowledge, little information is available about this triple investigation in colorectal cancer. Several questions remain with regard to the significance of circulating p53 antibodies. Does monitoring p53 antibodies really help physicians to manage patients with colorectal cancer? How well do these three assays perform with respect to their sensitivity and specificity?

The 41 sera of patients with colorectal adenocarcinoma were tested and 10 (24%) were positive for p53 antibodies in our experimental conditions. Only 10 of the 20 patients who displayed p53 overexpression had p53 antibodies in serum. A recent study (19) reported detection and monitoring of serum p53 antibodies in 54 patients with colorectal cancer. The authors correlated the presence of circulating antibodies to the accumulation of p53 protein in tumor cells and reported the ability of this serum test in the follow up of patients.

Despite the methodological differences between the present study and that of Hammel et al, (19) (method of p53 antibodies quantification, threshold value for p53 overexpression), the prevalence of p53 antibodies in colorectal cancer (24%) and the ratio of patients positive for p53 antibodies and p53 overex-

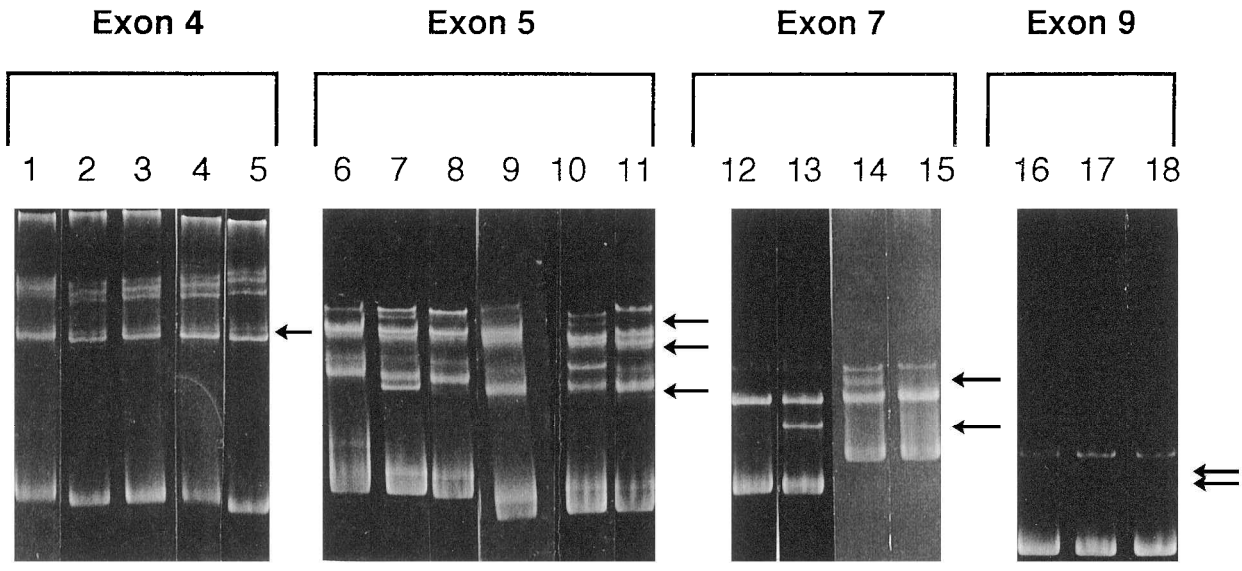

Fig 3. SSCP patterns of PCR-amplified exons of the TP53 gene. SSCP experiments were performed on the different amplified exons from the TP53 gene as described in Materials and Methods. Exon 4: lane 1: control; lanes 2–5: patients 4, 36, 23, and 34. Exon 5: lane 6: control; lanes 7–11: patients 32, 23, 5, 34, and 4. Exon 7: lanes 12 and 15: controls; lanes 13 and 14: patients 12 and 27. Exon 9: lane 16: control; lanes 17 and 18: patients 5 and 34. Bands with shift in mobility are indicated with arrows.

pression (10/20) were similar. In studies of other cancer sites, the prevalence of p53 antibodies was reported to be around 25% (8, 9, 11, 20–24).

p53 antibody response may have several explanations: (1) overexpression of an unaltered p53 protein; (2) emergence of a mutant nonaccumulated p53 protein; and (3) overexpression of a mutated protein. Our results show that p53 antibodies were associated with unaltered overexpressed p53 protein in 2/10 cases and overexpressed mutated p53 protein in 8/10 cases. As mentioned by Hammel et al (19) and Winter et al (12), we confirmed that the level of p53 overexpression did not seem to correlate with p53 antibody production: 10 patients were positive for p53 accumulation (50–100%), and none developed p53 antibodies. There may be several reasons: (1) the p53 protein may be stabilized by binding to other proteins such as *Mdm2* (25); (2) excessive amounts of normal protein are detectable by immunohistochemistry (26, 27); and (3) the immune status of the patients may also be involved (3).

Discrepancies between the presence of p53 antibodies and immunohistochemistry or between p53 antibodies and SSCP or DGGE analysis have been previously described (19, 23, 28–30). In the present study, none of the 10 patients with p53 antibodies showed any discordant result between serum p53 antibodies and immunohistochemistry. As reported in recent studies (1, 31), p53 mutations may occur outside exons 5–8. Our work provides information about mutations in exons 4 and 9. Of the 10 patients examined by SSCP analysis, two patients who were positive for serum p53 antibodies and p53 overexpression, were not SSCP positive. The sensitivity of SSCP analysis is still a matter of debate. In addition, numerous studies focused on exons 5–8, and negative SSCP results may occur because of an incomplete study of the TP53 gene. Although a complete study of the gene was performed at 4°C in our work, two samples with p53 overexpression and positive p53 antibodies failed to display any mutation by SSCP. The problem of false negative results with SSCP analysis remains. Experimental conditions such as temperature, ionic strength, and presence or absence of glycerol may be involved. The same finding has been described by Volkmann et al (21) and Wild et al (29): they noted false negative results with DGGE analysis. As reported by previous studies (29, 32), the problem of cell heterogeneity in the tumor sample remains: cells carrying a mutation may not be sufficiently represented in the tissue used for DNA extraction.

To date, CA 19-9 and CEA are used by physicians as routine tumor markers for colorectal cancer. p53 antibodies do not seem to correlate with CEA and CA 19-9 values in the preoperative period and during follow-up (data not shown). In our study, of the 41 patients tested, 13 (32%) showed an increase in CEA and/or CA 19-9. Only two patients of the 13 positive for CEA and/or CA 19-9 had p53 antibodies (15%). Seven of 23 patients (30%) developed p53 antibodies

with negative values of CEA and CA 19-9. These results are in close agreement with those reported by Hammel et al (19), but they are not consistent with recent observations from Shibata et al (30), who showed that the anti-p53 antibody test (immunoblotting method) was positive in 78% of patients with high serum carcinoembryonic antigen levels. Our study suggests that p53 antibodies may be useful for patient monitoring, especially when other tumor markers are normal. Concerning p53 antibody monitoring, if they were undetectable before surgery, we confirmed that they do not appear in serum during the follow-up, as previously shown (8, 10, 19).

Repeated testing for p53 antibodies showed a decrease in p53 levels in six patients, including the patient with liver metastasis who underwent palliative resection. Two of them returned to normal p53 antibody values. Two patients displayed stable and elevated p53 antibody concentrations, one of them with a probable correlation with an ovary tumor. The other one showed an increase in p53 antibody levels at three months, in relation to a probable progression of the disease. The patient with liver metastasis displayed a decrease in p53 antibodies (of 32%) after segmental hepatic resection. In the present study, patients whose levels of p53 antibodies returned to normal concentrations were those where initial values were low. The management of 10 patients, using p53 antibodies in a follow-up of 12 months, is too short to draw any conclusions about the prognosis of these patients. The presence of p53 antibodies may identify subgroups of patients with a poor prognosis, as was previously described (33).

Our results demonstrate the interest of testing for p53 antibodies in the serum of patients with colorectal cancer. We confirm that detection of circulating p53 antibodies provides valuable information about alterations in the TP53 gene. Along with CEA and CA 19-9, serological analysis of p53 antibodies constitutes a useful and noninvasive technique for the management of patients under treatment for colorectal carcinoma. The combination of CEA, CA 19-9, and circulating p53 antibodies would improve the sensitivity for clinical use.

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