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HUMORAL IMMUNE RESPONSE AGAINST P53 PROTEIN IN PATIENTS WITH COLORECTAL CARCINOMA

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***p53* aberrations are frequent in colorectal carcinogenesis (40–70%). Because *p53* gene mutations typically result in increased p53 protein concentration in tumor cells, this cellular protein might become immunogenic during tumor development. To test this hypothesis, serum p53 antibodies were quantitatively analyzed in 229 patients with colorectal cancer, using an immunofluorometric procedure. Circulating antibodies against p53 were found in 23% (53/229) of the patients. We quantified antibody concentrations in all positive sera and found that they varied from 300 to 500,000 arbitrary units/l. Sequential analysis of positive sera from 3 patients showed that p53 antibody concentrations change during the course of the disease, reflecting progression or regression. No association was found between the presence of p53 antibodies and age, sex, stage, histological grade and patient relapse-free or overall survival. These data demonstrate that antibody generation against the p53 tumor-suppressor protein is a relatively common event in colorectal cancer and that serological analysis for p53 antibodies may have some value for patient monitoring. The test has no value for prognosis. *Int. J. Cancer,* 70:46–51, 1997.**
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In the last few years, one of the most exciting and promising fields in cancer research has been the study of the biological and biochemical functions of the *p53* gene and protein. Although discovered 16 years ago, this molecule attracted interest only after 1990, when its role as a tumor-suppressor became clear (Finlay *et al.*, 1989; Baker *et al.*, 1990). Currently, *p53* is considered to be the most frequently mutated gene in human cancer (Caron de Fromentel and Soussi, 1992).

Of all carcinomas, about 50% contain mutations at the *p53* locus. Usually, one allele of the *p53* gene sustains a point mutation and the other one is lost. The various mutations are not randomly distributed throughout the gene but appear to be clustered between exons 5 and 8, which encode for highly conserved regions of the protein. These mutations lead to the production of an abnormal protein, which accumulates in the cancer cells presumably because of its increased stability. The accumulated mutant p53 can be detected in tumor sections by immunohistochemical methods.

Some tumors can induce the host to develop antibodies against mutant p53, most probably through loss of tolerance, due to abnormal accumulation of the defective protein. This was originally observed by Crawford *et al.* (1982), who detected circulating p53 antibodies in patients with breast cancer, followed by the work of Caron de Fromentel *et al.* (1987), who found that such antibodies were present in the sera of children bearing a wide variety of tumors. Later, it was demonstrated that the development of p53 antibodies is dependent upon complexing of the mutant protein with heat shock protein 70 (HSP 70) (Davidoff *et al.*, 1992) and is associated with the type of mutation (only mis-sense mutations give rise to an immunogenic protein) (Winter *et al.*, 1992). Another group has shown that p53 antibodies are directed toward immunodominant epitopes localized in the amino- and carboxy-terminal end of the protein (Schlichtholz *et al.*, 1992, 1994; Lubin *et al.*, 1993). The same group also demonstrated a correlation between the presence of such antibodies and other poor prognostic factors (high histological grade and absence of steroid hormone receptors) in breast cancer patients (Schlichtholz *et al.*, 1992). This correlation was later confirmed by others, who showed that p53 antibodies in the serum correlated positively with high histological grade and a history of second primary cancer (Mudenda *et al.*, 1994). We have performed an extensive study on p53 antibody generation in various cancers and found that the most immunogenic tumors are those of the colon and ovary (Angelopoulou *et al.*, 1994). In this report we have studied the immune response against p53 in patients with colorectal cancer.

MATERIAL AND METHODS

Patients

Serum samples from 229 patients with colorectal cancer were collected within 1 year from diagnosis and stored at −70°C until analysis. Previous work has shown that anti-p53 antibody concentrations are stable for at least 3 years at −70°C.

Cell line

The colorectal carcinoma cell line COLO 320 HSR(+) was obtained from the ATCC (Rockville, MD). This cell line expresses high levels of p53 as a result of a mutation at codon 248 (Murakami *et al.*, 1991; Hassapoglidou *et al.*, 1993). Cells were grown in suspension in RPMI culture medium containing 10% FCS and antibiotics.

Antibodies

The mouse monoclonal antibody (MAb) anti-p53 PAb240, which recognizes an epitope on the mutant p53 protein between amino acids 213 and 217, was used to capture the p53 antigen and immobilize it on the microtiter plate. The antibody was produced in our laboratory from a hybridoma cell line donated to us by Dr. D.P. Lane, University of Dundee, UK. The human anti-p53 antibodies in the serum were detected either with a goat anti-human IgG antibody conjugated to alkaline phosphatase (for the immunoassay procedure) or with a goat anti-human IgG antibody conjugated to horseradish peroxidase (HRP) (for Western blot analysis). Both secondary antibodies were purchased from Jackson Immunoresearch (West Grove, PA). The rabbit polyclonal anti-p53 antibody CM-1 (Dimension Labs, Mississauga, Canada) was used for detection of the p53 protein on the immunoblot, which was then recognized by a donkey anti-rabbit antibody conjugated to HRP. The mouse anti-p53 DO-7 MAb was used for immunohistochemistry. This antibody recognizes an epitope which resides between amino acids 35 and 45 of the p53 protein and reacts with both wild-type and mutant forms of the protein (Vojtesek *et al.*, 1992).

Immunoassay

For the quantitative analysis of p53 antibodies in serum, we used a time-resolved immunofluorometric technique, described in detail elsewhere (Angelopoulou and Diamandis, 1993). The method is based on measurement of alkaline phosphatase activity with a detection methodology which involves the alkaline phosphatase substrate 5-fluorosalicylphosphate, Tb^{3+} and EDTA. The principles

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of the procedure have been previously described (Christopoulos and Diamandis, 1992).

Quantification

Because of the lack of a suitable standard solution, we devised an arbitrary but comparative system to calibrate the method (Angelopoulou and Diamandis, 1993). One positive serum was assigned a p53 antibody concentration of 20,000 U/l. This serum was used in dilutions to construct calibration curves from which the concentrations of all other positive sera were calculated.

Assay for carcinoembryonic antigen (CEA)

For the analysis of CEA, we used the commercially available Amerlite CEA-60 assay (Kodak Clinical Diagnostics, Rochester, NY).

Protein A affinity chromatography

Protein A affinity chromatography was performed manually using the kit system MAPS, purchased from Bio-Rad (Richmond, CA). The instructions of the manufacturer were followed throughout.

High performance liquid chromatography (HPLC)

HPLC was performed with a Shimadzu (Kyoto, Japan) system with an absorbance monitor at 280 nm. The mobile phase was a 0.1 mol/l Na_2SO_4, 0.1 mol/l NaH_2PO_4 solution, pH 6.8. The flow rate was 0.5 ml/min and the HPLC was run isocratically. The gel-filtration column was a Bio-Sil SEC-250 column, 600 × 7.5 mm (Bio-Rad). The column was calibrated with an m.w. standard solution from Bio-Rad, containing thyroglobulin (670 kDa), IgG (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and cyanocobalamin (1.4 kDa). HPLC fractions (0.5 ml) were collected and individually analyzed.

Western blot

Lysates from COLO 320 HSR(+) cells were mixed with an equal volume of Tris-glycine-SDS buffer containing 2-mercaptoethanol, denatured by heating at 90°C for 5 min and loaded onto 8–16% polyacrylamide mini-gels (NOVEX, San Diego, CA). After electrophoresis (125 V, 90 min), the proteins were transferred to a nitrocellulose membrane (Hybond-ECL; Amersham, Arlington Heights, IL) by electroblotting at 30 V for 2 hr. The membrane was then treated overnight in a blocking solution (5% non-fat dried milk in wash solution [Tris-buffered saline, pH 7.6, 0.1% Tween-20]) and subsequently cut into strips which were probed for 1 hr at room temperature with the human sera (diluted 1,000-fold in a 6% [w/v] BSA solution) or with the rabbit polyclonal CM-1 anti-p53 antibody (diluted 1,000-fold in a 6% BSA solution). After washing with wash solution, the blot was incubated for 1 hr with a goat anti-human IgG conjugated to HRP, in the case of the human sera, and with a donkey anti-rabbit IgG conjugated to HRP, in the case of the CM-1. After a final washing, antibody binding was visualized by chemiluminescence and captured on X-ray film, using the ECL-Western blot detection kit from Amersham.

Immunohistochemistry

Sections (4 μm) were cut and placed on sialin-coated slides from formalin-fixed, paraffin-embedded tissue. The paraffin was removed in xylene, and the sections were rehydrated through graded concentrations of alcohol. Slides were placed in a thermoresistant plastic container filled with 10 mmol/l citrate buffer, pH 6.0, and processed in the microwave oven 5 times for 5 min each, at 750 W. Sections were allowed to cool down in the container at room temperature for about 20 min and then rinsed in TBS (50 mmol/l Tris, 150 mmol/l NaCl, pH 7.2). They were subsequently washed in blocking serum (5% normal goat serum) for 5 min and incubated with the primary antibody (DO-7) at 37°C for 1 hr. Slides were then washed in TBS 2 times and incubated with the secondary antibody (biotinylated goat anti-mouse antibody) at 37°C for 45 min. Slides were washed again 2 times in TBS and incubated at 37°C for 45 min with the avidin-peroxidase conjugate. The biotinylated antibody, avidin-HRP and substrate were components of the Vectastein ABC kit commercially available from Vector (Burlingame, CA). After another washing with TBS, slides were incubated for 4 min at room temperature with diaminobenzidine (DAB) solution (0.5% DAB in TBS, pH 7.6). After a final washing with water, slides were counter-stained with hematoxylin for 2 min, dehydrated, cleared and mounted.

Statistical analysis

The χ^2 test was used to determine the statistical significance of differences in distributions and all χ^2 values and the corresponding *p* values were calculated by SAS statistical software (SAS Institute, Cary, NC).

RESULTS

p53 antibodies in the sera of patients with colon cancer

Serum samples from 229 patients with colon cancer were analyzed for the presence of circulating p53 antibodies with a previously described immunofluorometric procedure (Angelopoulou and Diamandis, 1993). The presence of such antibodies could be demonstrated in 53 of the 229 sera (23.1%). Positivity was established when the fluorescence ratio obtained in the presence of p53 antigen and in the absence of p53 antigen (blank) was 1.7 or higher, as previously described (Angelopoulou and Diamandis, 1993). Analysis of 230 sera from non-cancerous individuals gave ratios less than 1.7 in 228 sera (data not shown). The other 2 sera had ratios below 4.

We quantified the p53 antibodies in all 53 positive sera using an arbitrary primary standard (Fig. 1). Antibody titers varied from a few hundred to 500,000 U/l. For most patients multiple samples, taken at different times during the course of the disease, were analyzed. The differences between titers in samples from the same patient were quite significant in some cases. For example, for patients 20 and 36 (Fig. 1) the range of antibody concentrations was greater than 10-fold (see also below). Importantly, none of the sera from any positive patient became negative later during the course of the disease, and similarly none of the sera from negative patients became positive. Thus, none of our patients could be misclassified due to variable sampling times after diagnosis. Our serum sampling was performed within 1 year from diagnosis in all patients.

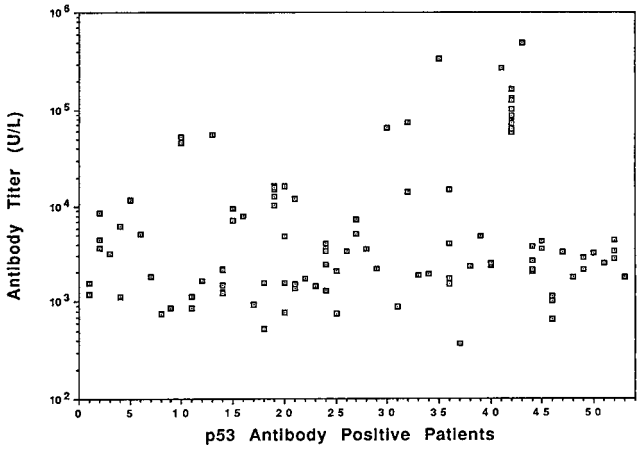

FIGURE 1 – Concentration of p53 antibodies in the sera of p53 antibody-positive colon cancer patients. Many patients have more than one serum sample (taken at different times during the disease). All antibody concentrations are expressed in arbitrary U/l.

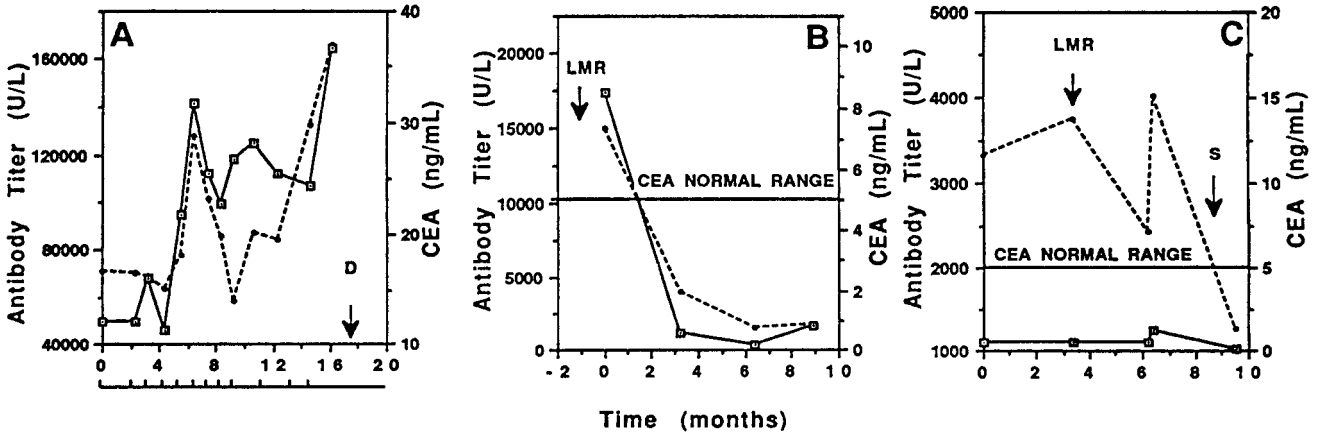

FIGURE 2 – Monitoring three colon cancer patients with CEA (solid lines) and with p53 antibody titers (broken lines). Vertical lines below the *x* axis in (*a*) indicate the times of chemotherapy administration. D, death; LMR, liver metastasis resection; S, surgery.

Changes of p53 antibody titers with disease course

We investigated whether the p53 antibody concentration in sequential sera changes with disease progression or regression. The results for 3 representative patients are shown in Figure 2. Similar data were obtained with another 3 patients, with at least 4 sequential sera tested for each.

Patient A was a 68-year-old man diagnosed with moderately differentiated stage C carcinoma of the rectum. He was treated with surgery but relapsed 2 years later with metastasis to the liver. After a second surgery, he was treated with 5-fluorouracil and leucovorin for 14 months. Three months after the last chemotherapy he died. p53 antibody and CEA concentrations were monitored in 13 consecutive samples, the first one taken 3 years after his first surgery (time 0). Patient B was a 60-year-old man diagnosed with moderately differentiated stage B sigmoid colon cancer. He was treated with surgery but operated 15 months later to remove a liver metastasis. No chemotherapy was administered. p53 antibody and CEA concentrations were monitored in 4 consecutive serum samples post-surgery for liver metastasis. Patient C was a 73-year-old woman diagnosed with moderately differentiated stage C sigmoid colon cancer. Consecutive serum samples were available before (1 sample) and after her liver metastatic tumor was resected.

The results shown in Figure 2 and similar data from another 3 patients suggest that the temporal changes of p53 antibody concentrations and CEA follow a similar pattern. Antibody titers seem to reflect tumor volume because they decrease after surgery and increase after relapse. Patient C had a tumor which did not produce CEA; thus, she could not be monitored with this tumor marker. Serum p53 antibody concentrations, however, were elevated and fluctuated according to the disease status.

Identification of anti-p53 antibodies

To confirm that the molecules detected with the immunofluorometric procedure are human immunoglobulins, we selected 15 sera and purified them by protein A affinity chromatography. These samples were 5 highly positive sera with p53 antibody titers between 100,000 and 500,000 U/l, 5 sera with titers between 10,000 and 20,000 U/l and 5 sera which were p53 antibody-negative. All were passed through a protein A column, which is known to bind only immunoglobulins. After elution, the eluates were analyzed with the immunofluorometric method. Anti-p53 antibodies were detected at similar concentrations before and after protein A purification, confirming that the analytes measured are indeed immunoglobulins. No p53 antibodies were detected in the immunoglobulin fractions of sera negative for p53 antibodies.

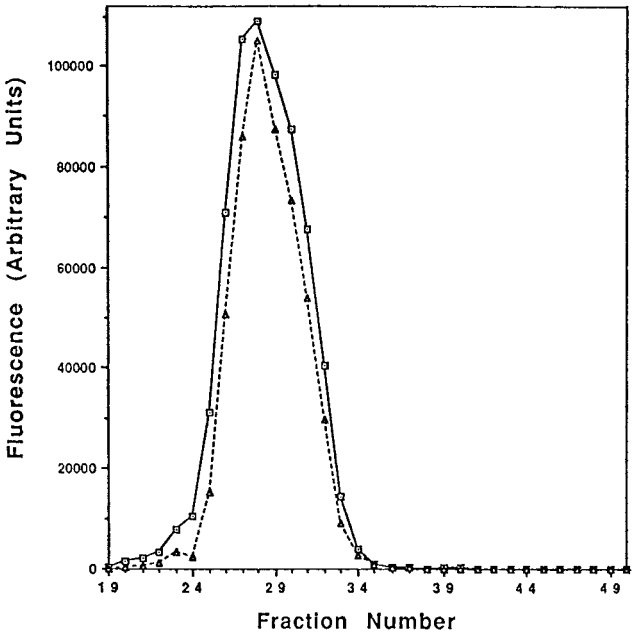

FIGURE 3 – Fractionation of a p53 antibody-positive serum before (solid line) and after (broken line) purification with protein A affinity chromatography, with gel filtration HPLC. Fractions were analyzed for p53 antibodies by the immunofluorometric procedure. The peak elutes at a fraction corresponding to an m.w. of 160–180 kDa, which is consistent with the m.w. of human immunoglobulins.

Some sera positive for p53 antibodies were separated on an HPLC system using a gel filtration column. The chromatographic fractions were analyzed for p53 antibodies by the immunofluorometric assay. Positive HPLC fractions corresponded to an m.w. consistent with human immunoglobulins (160–180 kDa). Data for one of the sera analyzed are shown in Figure 3.

Western blot analysis

To demonstrate the specificity of the serum auto-antibodies with the p53 protein, we performed immunoblots of positive and negative sera. COLO 320 HSR(+) cells were used as a source of the p53 protein. This colorectal carcinoma cell line expresses high levels of mutant p53. The cell lysate was separated on a polyacrylamide gel, transferred to nitrocellulose membrane and probed with

the human sera. The rabbit polyclonal anti-p53 antibody CM-1 was used as positive control. Data for some patients are shown in Figure 4. Positive samples immunoreacted with a 53 kDa protein; negative ones did not. These data demonstrate that the serum antibodies detected by the immunological assay are indeed specific for the p53 protein.

Immunohistochemistry

We investigated whether detection of p53 antibodies in serum is associated with accumulation of mutant p53 in the corresponding tumor. Immunohistochemical analysis was performed in tumor sections of 4 colon cancer patients. Three tumors were from patients positive for serum p53 antibodies and 1 from a negative patient. All 3 tumors from patients with p53 serum antibodies were positive for p53 protein, localized in the nucleus of tumor cells. The tumor from the patient with no serum p53 antibodies was negative for p53 protein (Fig. 5).

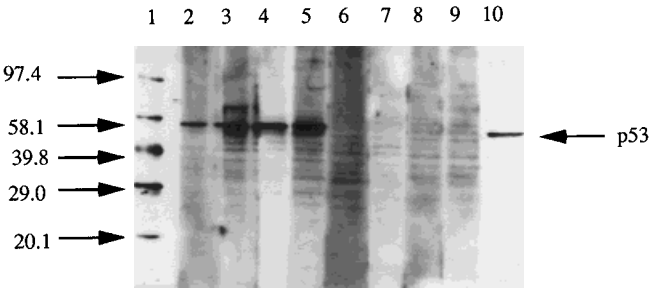

FIGURE 4 – Western blot analysis using human sera as sources of p53 antibodies. Lane 1: Molecular weight markers with m.w. in kDa shown. Lanes 2–10: Mutant p53 protein, extracted from the colorectal carcinoma cell line COLO 320 HSR(+), was separated on a polyacrylamide gel and transferred to a nitrocellulose membrane; p53 was then reacted with serum samples diluted 1,000-fold (lanes 2–9) or the polyclonal anti-p53 antibody CM-1 (lane 10). Lanes 2–5: Four different serum samples from patients with colon cancer, positive for p53 antibodies. Lanes 6–9: Four different serum samples from patients with colon cancer, negative for p53 antibodies. Lane 10: Detection with the rabbit polyclonal p53 antibody CM-1 (positive control).

Association between p53 antibodies and clinicopathological variables

We investigated the association between the presence of circulating p53 antibodies and other clinical parameters (Table I). No association was found between the presence of such antibodies and

TABLE I – ASSOCIATIONS BETWEEN p53 ANTIBODIES AND VARIOUS CLINICOPATHOLOGICAL VARIABLES IN COLON CANCER PATIENTS

Variable	Antibody-positive		Antibody-negative		*p* value
	Number	%	Number	%	
Age (yr)					
<60	6	11.8	31	17.4	
60–69	17	33.3	53	29.8	
70–79	15	29.4	73	41.0	
≥80	13	25.5	21	11.8	0.06
Sex					
Male	30	58.8	106	59.6	
Female	21	41.2	72	40.4	0.93
Grade[1]					
I	0	0	12	10.4	
II	35	97.2	93	80.9	
III	1	2.8	10	8.7	0.05
Stage[2]					
A	6	14.6	17	11.6	
B	17	41.5	80	54.4	
C	14	34.2	33	22.5	
D	4	9.8	17	11.6	0.37
Relapse[3]					
Yes	11	26.8	32	22.7	
No	30	73.2	109	77.3	0.58
Death[4]					
Yes	4	10.5	16	12.1	
No	34	89.5	116	87.9	0.79

[1]Information not available for 78 patients.–[2]Information not available for 41 patients.–[3]Information not available for 47 patients.–[4]Information not available for 59 patients.

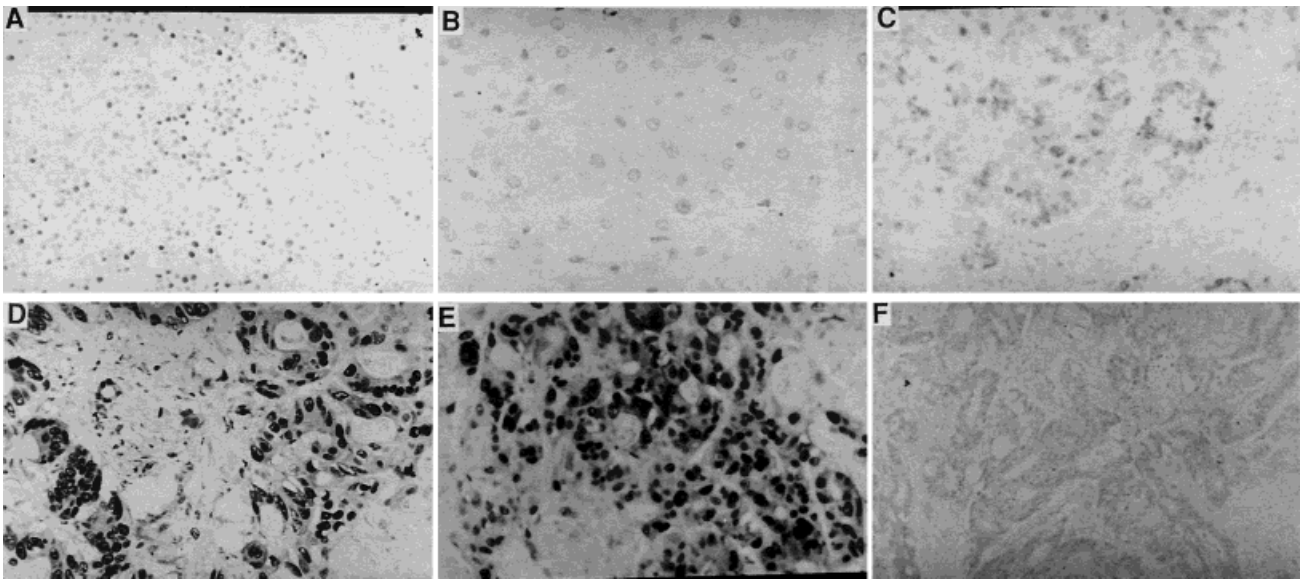

FIGURE 5 – Immunohistochemical analysis of tumor sections. (*a*) p53-positive control. (*b*) p53-negative control. (*c*) Colon cancer section positive for p53; the corresponding serum was positive for p53 antibodies. (*d, e*) Tumor sections from 2 different patients with metastatic colon adenocarcinoma to the liver, positive for p53; the corresponding serum samples were positive for p53 antibodies. (*f*) Colon cancer section negative for p53; the corresponding serum was negative for p53 antibodies.

age, sex, histological grade and stage. Patients for whom antibodies were detected did not relapse or die with a different frequency from those who did not develop antibodies. These data suggest that p53 antibodies do not have a prognostic value for colon cancer patients.

DISCUSSION

p53 is mutated in 40–70% of colorectal carcinomas (Soussi *et al.,* 1994). In most cases, one allele of *p53* has a point mutation, while the other one is lost *via* gene conversion or deletion. Point mutation of *p53* often leads to a protein with increased stability, which accumulates in the malignant cell and is detectable immunohistochemically. In normal cells, the protein is practically undetectable.

Although over-expressed in the tumor, mutant p53 has not been detected in the serum of cancer patients (Hassapoglidou *et al.,* 1993). However, antibodies against this protein are released into the circulation and can be detected in the serum by Western blot (Caron de Fromentel *et al.,* 1987; Davidoff *et al.,* 1992; Winter *et al.,* 1992; Schlichtholz *et al.,* 1992) or by immunoassay techniques (Lubin *et al.,* 1993; Schlichtholz *et al.,* 1994; Mudenda *et al.,* 1994; Angelopoulou *et al.,* 1994; Angelopoulou and Diamandis, 1993). The humoral immune response against p53 has been studied only in patients with breast (Crawford *et al.,* 1982; Davidoff *et al.,* 1992; Schlichtholz *et al.,* 1992; Mudenda *et al.,* 1994) and lung (Winter *et al.,* 1992; Schlichtholz *et al.,* 1994) cancers.

We investigated the antigenic p53 response in patients with colon cancer; 23% (53/229) of patients developed antibodies. The diagnostic specificity of the p53 antibody test was previously evaluated by our group (Angelopoulou *et al.,* 1994) and others (Crawford *et al.,* 1982; Caron de Fromentel *et al.,* 1987; Davidoff *et al.,* 1992; Winter *et al.,* 1992; Mudenda *et al.,* 1994) and was found to be close to 100%.

We assessed the concentration of p53 antibodies in all positive sera (Fig. 1). Antibody titers varied considerably between patients (300–500,000 U/l). These large differences between p53 antibody levels among patients as well as the finding that only a proportion of patients with mutant p53 in their tumors develop antibodies have not as yet been clearly explained. Davidoff *et al.* (1992) have shown that tumors which elicit an antibody response contain complexes between HSP 70 and mutant p53. Winter *et al.* (1992) found that only tumors in which the *p53* gene bears mis-sense mutations are able to induce antibodies and that not all patients with *p53* mis-sense mutations develop these antibodies. In colon cancer, various studies have reported that the *p53* gene is mutated in 40–70% of cases (Soussi *et al.,* 1994; Hollstein *et al.,* 1991). Our data support the view that about half of the patients with a mutated *p53* gene develop p53 antibodies (23%).

We investigated changes in p53 antibody serum concentrations during the course of colon cancer (Fig. 2). We found that antibody levels increase in case of relapse and decrease after surgery or other therapeutic manipulations. However, these antibodies, unlike some other tumor markers, do not disappear from the circulation. They may be present in serum for months or years even in patients in remission, but their titers change during the disease.

Protein A affinity chromatography and HPLC were performed to confirm that the moieties detected in the human sera by the immunological assay were human immunoglobulins (Fig. 3). The specificity of these immunoglobulins for p53 protein was further tested with Western blot analysis (Fig. 4). The results showed that sera positive for p53 antibodies, but not sera-negative for p53 antibodies, react with a 53 kDa protein, which is also visualized with a specific polyclonal anti-p53 antibody.

A few tumors were tested immunohistochemically (Fig. 5). Patients with p53 antibodies in their serum had tumors displaying p53 accumulation.

We have also investigated whether there is any association between the presence of p53 antibodies and other clinicopathological variables in colon cancer patients. In accordance with the results of Caron de Fromentel *et al.* (1987) and Winter *et al.* (1992), who studied p53 antibodies in sera of children with B-cell lymphoma and patients with lung cancer, respectively, we could not establish any association between the presence of p53 antibodies and patient age, sex, stage, histological grade, relapse or survival.

It is not yet known at which stage during tumor development p53 antibodies appear in the serum. Our limited data suggest that these antibodies are present with equal frequency in early- and late-stage disease. If indeed such antibodies appear early in tumor development, they could offer a basis for early diagnosis of a small subset of colon cancer patients with a simple and non-invasive test (Angelopoulou *et al.,* 1994). We are currently investigating if such antibodies appear in pre-malignant colon pathologies and in patients who visit colon cancer clinics for the first time.

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