p53 Antibodies in Patients with Various Types of Cancer: Assay, Identification, and Characterization

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Received 4/10/95; revised 7/19/95; accepted 7/28/95.

Abstract
Alteration of the p53 gene is the most frequent genetic alteration in human cancer and leads to the accumulation of mutant p53 in the nucleus of tumor cells. In addition, it has been shown that patients with various types of neoplasia have p53 antibodies in their sera which could be used as an indirect diagnostic procedure for p53 alteration.

Using a new ELISA, we have analyzed the sera from more than 1000 patients with various types of cancer and from healthy blood donors. We demonstrate that p53 antibodies are detected mainly in cancer patients and are strictly proportional to the occurrence of p53 mutations. Using various immunological approaches, these antibodies were unambiguously demonstrated to be directed toward the human p53 protein. Isotyping analysis of these antibodies strongly suggested that they correspond to a humoral response to the p53 protein which accumulates in the tumor cell. This finding suggests that serological analysis, combined with histochemistry, is suitable for assessing the integrity of the p53 gene in cancer patients.

Introduction
Inactivation of the p53 tumor suppressor gene is the most common genetic alteration in human cancers, and may occur through point mutations or complex formation with cellular proteins (1, 2). The recognition of the p53 alteration is an important feature in clinical diagnosis, since it is an independent, unfavorable prognostic factor in breast, colon, and gastric carcinomas (3–5). Furthermore, it has been shown that the vulnerability of tumor cells to radiation or chemotherapy is greatly reduced by mutations that abolish p53-dependent apoptosis (6). Analysis of p53 status by DNA sequencing is the most accurate method described thus far, but it is not easy to use on a routine clinical basis for a large number of samples. Fortunately, it is known that most mutations induce an increase in the p53 half-life, leading to an accumulation of p53 protein in the nucleus of tumor cells. Immunohistochemical analyses have been extensively used for the screening of the p53 alteration in a wide variety of human cancers (7) for review. More recently, a serological method has been proposed for diagnosis of the p53 alteration. p53-Abs have been detected in sera of patients with various carcinomas (8–16). The presence of these antibodies was shown to be dependent on the accumulation of p53 protein in tumor cells (10, 12, 17), but some exceptions have been reported (15, 16). In breast carcinoma, the presence of p53-Abs is correlated with poor prognostic factors such as high histological grade and the absence of hormone receptors (11, 18, 19). A more recent study on 400 patients with breast carcinoma demonstrated that the overall survival was worse in patients with p53-Abs (19). In lung cancer, p53-Abs can be detected several years before clinical diagnosis of the tumor (20). Analyses of this humoral response have demonstrated that p53-Ab recognizes immunodominant regions localized in the amino and carboxy termini of the p53 protein, outside the mutational hot spot (11, 21, 22).

In addition, serological analysis of p53 can be used as a complementary procedure with molecular and immunohistochemical methods, since it does not require tumor tissues and can be easily used for follow-up of patients with p53 alterations (7).

In light of various reports, the frequency of these p53-Abs for a given cancer remains a matter of debate. In breast carcinomas, which have been extensively studied, it may range from 1 (23) to 5% (13), 9% (8), 14% (11, 19), and 25.6% (18). This discrepancy can be partially explained by the various techniques (ELISA, immunoprecipitation, or Western blot) used in these studies, but it might also reflect some unsuspected bias in the choice of patients (difference in clinical status, environmental, or geographical factors). More surprising was the report by Vojtesek et al. (23) describing the near total absence of p53-Abs (1:100) in sera of patients with breast carcinoma. These authors raised the possibility that some of the previously described p53-Abs could be directed toward an unknown Mr 53,000 protein unrelated to p53.

In the present report, we describe a novel ELISA for the detection of p53-Abs in human sera. Using conventional immu-

The abbreviations used are: p53-Ab, autoantibodies to p53; PBS-T, PBS with 0.05% Tween 20; SD, standard deviation.
nological methods, we demonstrate that these antibodies are directed against the p53 protein. We show that the prevalence of p53-Abs is correlated with the prevalence of p53 mutations in the different cancer types. Isotyping of these antibodies has demonstrated that most of them belong to the IgG subclass, reinforcing the notion that this humoral response is the result of an active self-immunization process.

Materials and Methods

Sera and Antibodies. All sera were collected from various clinical laboratories in France between 1992 and 1994. They were obtained after diagnosis, but prior to any treatment. Sera were stored at -70°C until use. Sheep antihuman IgG peroxidase-conjugated antibodies (γ chain specific and affinity isolated; Silenius GAH) were used for the detection of human p53 antibodies in the ELISA. To evaluate the subclasses of immunoglobulin specific for the p53 protein, the following mouse monoclonal antibodies were used: HP6001 (IgG1 subclass), HP6002 (IgG2 subclass), HP6050 (IgG3 subclass), HP6024 (IgG4 subclass), DA4.4 (IgM subclass), and 2D7 (IgA1 + 2 subclass; Ref. 24). The specificity of each monoclonal antibody was assessed using the pure immunoglobulin subclass. Mouse monoclonal antibodies were detected using a goat antimouse IgG (γ chain specific and human adsorbed; Caltag Laboratories, M30107). HR231, a mouse monoclonal antibody which recognizes human p53, has been previously described (25).

Immunoblotting and Immunoprecipitation. For immunoblotting, we used wild-type human p53 protein expressed in insect cells; 48 h after infection by the recombinant baculovirus, cells were lysed using RIPA [150 mm NaCl, 10 mm Tris-HCl (pH 8.0), 1% sodium deoxycholate, 0.1% sodium lauryl sulfate, 1 mm EDTA] + SDS buffer for 30 min at 4°C. The extract was centrifuged for 30 min at 100,000 g, and the supernatant containing soluble p53 was stored at -80°C until use. p53 protein corresponded to 20–40% of the total protein content in our conditions. For the ELISA, a control extract prepared from cells infected either with wild-type virus or with a recombinant virus encoding a protein unrelated to p53 was used. For competition experiments, human p53 protein was purified by immunoaffinity using monoclonal antibody HR231. As judged by staining and immunoblotting, the p53 protein was more than 95% pure. Immunoblotting of human sera has been previously described (11). For immunoprecipitation, full-length wild-type p53 was obtained by in vitro transcription/translation. For each immunoprecipitation, 10,000 cpm of labeled protein were used as described by Soussi et al. (26).

ELISA. Polystyrene flat-bottomed microtiter plates (Immulon B; Dynatech Laboratories) were coated with 50 μl p53 extract (corresponding to 40–80 ng p53 protein) in PBS buffer. The same cell extract was used for all of the experiments described in this report. Plates were dried for 48 h at 37°C and then sealed in a polypropylene bag and stored at 4°C until use. Such plates give reproducible results over a period of 6 months. Before use, plates were washed five times with PBS-T; 100 μl blocking buffer (PBS-0.2% Tween 20, and 5.0% dried nonfat milk) were added per well. After 1 h at 37°C, the wells were washed as described above. Then 50 μl sera (diluted 1:50 and 1:100 in PBS, 5.0% dried non-fat milk) were tested in duplicate. The plates were incubated for 1 h at room temperature on an ELISA plate shaker. After five washings in PBS-T, 100 μl of an antihuman IgG peroxidase conjugate diluted 1:2500 in PBS-5% nonfat milk were added and incubated for 1 h at 37°C. Preliminary experiments were performed with an antihuman immunoglobulin antibody, but since we had demonstrated that all of these p53-Abs include IgG, we used an antihuman IgG antibody. Plates were then washed five times and developed using 2,2′-azino-di(3-ethylbenzthiazoline) sulfonate substrate in citrate buffer (Boehringer, catalogue no. 1204521). Absorbance was read at 405 nm after 30 min using an MR 5000 ELISA reader (Dynatech Laboratories). All of these manipulations were performed simultaneously on a plate coated with either p53 protein as described above or with the control extract. Each serum was tested by duplicate replication at two different dilutions (1:50 and 1:100). SDs were calculated using the four wells.

For the competition experiment, the ELISA procedure was similar with the following exception: prior to ELISA, the sera (diluted 1:100 in PBS-5% nonfat milk) were incubated with various amounts of either purified p53 or ovalbumin (as a negative control). Incubation was performed in 96-well plates that had been washed with PBS-T prior to incubation. Plates were incubated for 60 min at room temperature on an ELISA plate shaker. Sera were then tested as described above. Competition experiments were also performed simultaneously on a plate coated either with p53 protein or with the control extract.

For isotyping the p53 antibodies, we used the protocol described above with the following modifications: only one dilution of sera was used (1:50 in PBS-5% nonfat milk). After incubation with the sera and washing, antihuman isotype-specific monoclonal antibodies were used to reveal the antibody-antigen complex; 50 μl of antibodies were added to the well and incubated for 1 h at 37°C. The wells were then washed five times with PBS-T, and 100 μl goat antimouse immunoglobulin peroxidase-conjugated antibodies (Caltag, catalogue no. M30107, human absorbed, diluted 1:3000) were added to each well. After 1 h at 37°C, plates were washed five times and bound antibodies were revealed by adding 100 μl 3.3′5′-tetramethylbenzidine substrate. The reaction was stopped after 10 min by adding 100 μl 1 M phosphoric acid. Plates were then read at 450 nm.

Results

A New ELISA for the Detection of Anti-p53 Antibodies. We have recently reported the presence of p53-Abs in patients with breast and lung cancers (11, 21). Sera were tested using Western blot or immunoprecipitation. To test several thousand sera and to set up an assay for routine diagnosis, we developed an ELISA for the detection of p53-Abs in sera. Preliminary experiments showed that human sera can lead to important, variable background signals (data not shown). This background was shown to be independent of the protein extract coated in the plate, and seemed to reflect a nonspecific interaction of serum components with either the plastic or components used in the ELISA. Thus, an ELISA was developed that used an internal control to measure the nonspecific background of each serum. As described in “Materials and Methods,” each serum was
tested simultaneously with a cell extract containing p53 and with a control extract which was devoid of p53. Theoretically, a normal serum should give a similar signal with the two extracts, leading to a ratio of p53:control very close to 1.0. This ratio is independent of the background of the serum. This assay was developed using sera from breast cancer patients which were previously shown to contain p53-Abs detected either by immunoprecipitation or Western blot. As shown in Fig. 1A, sera containing p53-Abs led to a ratio value higher than 2.0, whereas negative sera gave rise to a value of around 1.0. Fig. 1B shows a typical Western blot experiment using a cell extract from insect cells infected with a recombinant baculovirus expressing human wild-type p53. These sera did not detect any specific protein when incubated with control extract obtained from cells infected with a wild-type virus (data not shown). In Western blot experiments, the p53 was fully denatured, whereas in ELISA, the wild-type p53 was partially denatured during the coating procedure. Using immunoprecipitation of in vitro translated human wild-type native p53, we showed that the serum p53-Abs were also able to recognize native p53 (Fig. 1C).

Absence of p53-Abs in Sera from Healthy Donors. We tested 200 sera from healthy blood donors theoretically negative for p53 antibodies (Fig. 2). Each serum was tested in duplicate using two different dilutions (1:50 and 1:100). In agreement with our hypothesis, the mean ratio obtained with all of these sera was 1.1, with a SD of 0.4. Using a cutoff value corresponding to the mean plus 2 SDs (97.7% of confidence), all but one sera were negative in ELISA. This serum (X72) had a value (2.6 ± 1.5) which was reproducibly higher than this ratio. It was also tested by Western blot, and p53 antibodies were readily detectable using this method (Fig. 1D). Several samples of serum from the same woman over a period of 15 months were obtained from January 1992 to July 1993. All were positive, with a slight constant increase over this period. Examination of the clinical records did not show the presence of any neoplastic disorder. The woman was lost for continuous follow-up, and no further studies were performed on this case. Taken together, these data led us to conclude that the prevalence of p53 antibodies in the normal population is very low, and that our ELISA could be effectively used on a population with various types of cancer.

Prevalence of p53 Antibodies in Patients with Various Types of Cancer. The ELISA was then used to detect p53-Abs in sera of more than 1000 patients with various types of neoplasias (Fig. 2 and Table 1). Each experiment was performed using similar positive and negative controls. Positive controls included two sera. One of them (LC84) had an ELISA ratio of around 30, with p53-Abs recognizing p53 by both immunoprecipitation and Western blot (Fig. 1C). The second serum (BC20) also contained p53-Abs, as demonstrated by immunoprecipitation and Western blot (Fig. 1B). It had an ELISA ratio slightly higher than the cutoff value discussed above. This serum was used as a threshold for delimiting positivity. p53-Abs were found in the sera of patients with every type of neoplasm. The frequency ranged from 24% for lung cancer to 1% for prostate carcinoma (Table 1). Statistical analysis show that the presence of p53 antibodies is highly specific for cancer patients ($P < 0.001$).

Although the mechanisms involved in the appearance of these antibodies are not fully understood, it has been strongly suggested that they are associated with an alteration in the p53 gene which might lead to p53 accumulation. The rate of p53 antibodies found in our ELISA study were compared with the rate of p53 mutations described in the literature (Fig. 3).
was a striking correlation between the two rates, again suggesting that $p53$ mutations are involved in the appearance of these antibodies. This is consistent with the hypothesis that these antibodies are directed toward the $p53$ protein and are not the result of a cross-reaction with another cellular protein. Dilution experiments showed that the $p53$-Abs titer can vary from one patient to another. Fig. 1D shows a typical Western blot experiment using two sera, one (X72) from the healthy blood donor described above with a low titer of $p53$-Abs and one (I46) with a high titer of $p53$ antibodies and a negative serum (X2). Serum X72 scored positive until diluted 1:200, whereas serum I46 showed a very strong signal for a dilution of 1:50,000. This serum could be diluted 1:500,000 and still yield a positive signal (data not shown). Negative sera did not give any signal.

**Specificity of the Antibodies.** To ensure that these antibodies were really specific to $p53$, we performed ELISA competition experiments using either purified human $p53$ or ovalbumin as a control (Fig. 4). Only the purified $p53$ protein was able to compete, whereas ovalbumin did not induce any blockage. Neither $p53$ nor ovalbumin had any effect on the background signal obtained with the control extract. This experiment demonstrates unambiguously that these serum antibodies are directed toward human $p53$.

Experiments combining immunoprecipitation, Western blot, and ELISA on the same sera showed excellent correlation for the detection of serum $p53$-Abs (>90%), though it did not reach 100% (Fig. 1, A–C). Some sera were found to be positive in one or two assays only. It is difficult to reach a conclusion
some discrepancies concerning the frequency of these antibodies with either breast or lung cancer. Nevertheless, there are studies that have confirmed the presence of such antibodies in patients due to our lack of knowledge concerning p53 alterations. The absence of a signal, and the results could not be interpreted.

Isotype Analysis of p53 Antibodies. No isotypic analysis of p53-Abs has been reported. Thus, to better define the humoral response of cancer patients to p53, we analyzed the isotype of these p53-Abs (Fig. 5). A total of 28 patients were tested; 20 of them had been previously positive for p53 antibodies, while 8 were negative. The latter sera remained negative in the isotype-specific assay. Among the 20 positive sera, two gave a very weak signal, and the results could not be interpreted. Analysis of the other 18 patients showed that 15 sera contained mostly IgG1 and IgG2, while the 3 others exhibited a predominant IgA response. Several patients (6) also contained IgM, although none had p53-IgM as the only isotype. No IgG3 or IgG4 was detected. This result strengthens the hypothesis of an active humoral response against p53 and indicates that the presence of p53-Abs is not due to cross-reacting low-affinity IgM.

Discussion

p53-Abs were first described in sera of animals bearing tumors (27-29). Later, Crawford et al. (8), using immunoprecipitation, described the presence of p53-Abs in sera of breast cancer patients. Following this, Caron de Fromentel al. (9) found similar antibodies in the sera of children with B-cell lymphomas. These observations were ignored for several years due to our lack of knowledge concerning p53 alterations. The discovery that the p53 alteration can lead to p53 protein accumulation shed new light on these findings, suggesting that these p53 antibodies could be correlated with p53 alterations. Recent studies have confirmed the presence of such antibodies in patients with either breast or lung cancer. Nevertheless, there are some discrepancies concerning the frequency of these antibodies in breast cancer. It may range from 1% (23) to 5% (13), 9% (8), 14% (11, 19), and 25.6% (18). Furthermore, the nature of such antibodies has recently been questioned (23), and it has been proposed that another M, 53,000 protein could be the target for such antibodies. Most of these previous studies were performed on small series of samples using either immunoprecipitation or Western blot. In light of the possibility that such a test could be of clinical value, we have devised a simple ELISA assay enabling the easy, rapid screening of large numbers of samples.

We tested the sera of more than 1000 patients with various types of neoplasias and 200 healthy volunteers. We demonstrate here that p53-Abs are present primarily in patients with neoplasias. We show unambiguously that these antibodies are directed toward human p53: (a) the antibodies recognized the p53 protein using three methods (immunoprecipitation, Western blot, and ELISA), corresponding to the native or denatured state of p53; (b) the antibodies were specifically blocked by human p53 protein, whereas control protein did not abolish the reaction; and (c) the frequency of these serum p53 antibodies was correlated with p53 alterations in human cancers. In view of these results, along with our previous work showing that these p53-Abs recognized immunodominant epitopes localized in the amino and carboxyl termini of the p53 protein, and given the similarity of the immune response of patients and animals immunized with human wild-type p53, we conclude that these antibodies are directed toward human p53. All of these observations are strengthened by the observation that the presence of p53 antibodies is directly linked to several clinical prognosis markers (11, 19).

The most important question raised by the serological analysis concerns its correlation with the p53 mutation and/or p53 accumulation. Several studies have addressed this question (10, 12, 15-17, 22, 30). All of these data suggest that most patients with p53 antibodies have a p53 mutation which leads to p53 accumulation. Nevertheless, exceptions exist (15, 16): certain patients have p53-Abs, yet no p53 mutation is found in the tumor. We should emphasize that assay of p53 antibodies corresponds to a global approach to assessing p53 alterations and does not depend on sampling of the tumor, the composition of which may be very heterogenous. Molecular analysis of tumor

### Table 1  p53-Abs in patients with various types of neoplasias

<table>
<thead>
<tr>
<th>Neoplasia</th>
<th>p53 positive/total</th>
<th>% of patients with p53 antibodies</th>
<th>Frequency of p53 antibodies* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>10/42</td>
<td>24</td>
<td>60</td>
</tr>
<tr>
<td>Pancreas</td>
<td>14/73</td>
<td>19</td>
<td>44</td>
</tr>
<tr>
<td>Bladder</td>
<td>9/52</td>
<td>17</td>
<td>34</td>
</tr>
<tr>
<td>Breast*</td>
<td>14/106</td>
<td>13</td>
<td>22</td>
</tr>
<tr>
<td>Breast†</td>
<td>42/353</td>
<td>12</td>
<td>22</td>
</tr>
<tr>
<td>Breast‡</td>
<td>14/100</td>
<td>14</td>
<td>22</td>
</tr>
<tr>
<td>Thyroid</td>
<td>4/108</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>Leukemia</td>
<td>3/92</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Prostate</td>
<td>1/83</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Healthy donor</td>
<td>1/208</td>
<td>0.5</td>
<td>0</td>
</tr>
</tbody>
</table>

* The mutation data were compiled from Soussi et al. (7).
† Three series of breast carcinomas from three different hospitals were tested.
tissues or biopsies corresponds to local analysis of p53 status, and might be erroneous if the tumor is too heterogenous or too highly contaminated by normal tissue. Furthermore, mutation is not necessary for p53 accumulation (31, 32), and we have been able to detect p53 antibodies in such patients.6 However, it is also clear that not all patients with a p53 alteration develop p53 antibodies. Davidoff et al. (10) suggested that the type of mutation could influence the production of p53 antibodies, but given more recent results (16) this hypothesis requires further investigation. It is also possible that, for an identical mutation, the humoral response is dependent on the MHC class I or II molecule specific to each individual. If we compare the frequency of p53 alteration in the literature, the present work indicates that 30–40% of patients with an alteration in the p53 gene develop p53 antibodies. In a more recent work, using a new ELISA based on specific p53 peptides, we were able to detect p53-Abs in sera of nearly 40% of patients with p53 mutations.7

It has been proposed that the p53-Ab is the result of a self-immunization process toward an antigen which is normally expressed in minute quantities in the organism (22). This is supported by the finding that the immunoglobulin subclasses of these antibodies are characteristic of an immune response toward an antigen. Furthermore, the finding of IgG1 and IgG2 in all sera supports the notion that these p53-Abs correspond to a secondary response. Since all of these sera were taken at the time of diagnosis, this suggests that p53-Abs were present prior to the clinical manifestation of the cancer. In fact, we recently observed p53 antibodies in the sera of two heavy smokers several months before clinical detection of lung cancer (21).8

In a recently published study performed by Angelopoulou et al. (13), the authors tested over 1000 sera from patients with various types of cancer using a direct ELISA. They found p53-Abs only in patients with cancers, but the frequencies were lower than those described in the present work. This could reflect a bias in selection of the patient, but could also be due to the difference in the assay. At present, the status of the assay of serum p53-Ab is similar to that of the evaluation of p53 accumulation in tumor tissues several years ago. Numerous discrepancies were (and still are) observed due to the lack of standardization of the technique itself, but also to methods for recording the results. Assay of p53-Ab is still in its infancy because more data are needed to standardize the approach. Serological analysis of p53 alterations has several advantages, including follow-up of patients during treatment and early detection of p53 alterations. In view of the use of p53 as a new tumoral marker, the combined use of immunohistochemical and serological analyses should be a valuable asset in clinical investigations.

Fig. 4 | ELISA competition experiment. Prior to the ELISA, sera (1:100 diluted) were incubated with various amounts of p53 protein or ovalbumin (8, 40, and 160 ng/well). ELISA was then performed as described in “Materials and Methods” using either p53 (●, ○) or control antigen (■, □) on the plates. ELISA was performed either with ovalbumin (○, □) or p53 protein (●, ■). LC132, patient with lung cancer; TY68, patient with thyroid cancer; BC51, patient with prostate carcinoma; UC74, patient with bladder carcinoma; BC33, patient with breast carcinoma; X5, healthy donor.

Fig. 5 | Isotyping of p53 antibodies. UC132 and UC229, patients with bladder cancer; PC51, patient with prostate carcinoma; BC542, BC33, and BC77, patients with breast carcinoma; LC374 and LC132, patients with lung cancer.

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7 R. Lubin, I. Bouchet, and T. Soussi, manuscript in preparation.
8 Unpublished data.
Acknowledgments

We are grateful to K. Ory and Y. Legros for discussion, Y. Legros for his generous gift of purified p53 protein, B. Vojtesek and D. Lane for communicating their results prior to publication, and J. Bram for reading the manuscript.

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