Advances in Brief

Monitoring of p53 Autoantibodies in Lung Cancer during Therapy: Relationship to Response to Treatment

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Abstract

Alteration of the p53 gene is the most frequent genetic alteration in human cancer, and it 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 neoplasias have p53 antibodies in their sera. ELISA was used to detect anti-p53 antibodies in the sera of 167 patients with lung cancer. Among these, 32 individuals (16 positive for p53 antibodies and 16 negative) were monitored over a period of 30 months for p53 antibodies. Twelve of 16 antibody positive patients had reduced titers during chemotherapy that led to partial or complete remissions of disease. The specificity of these antibodies was confirmed by two different ELISA procedures and by immunoprecipitation. The very rapid, specific decrease in these antibodies during therapy suggests that a constant level of tumoral cells with nuclear accumulating p53 protein is necessary for a detectable humoral anti-p53 response. The good correlation found between the specific evolution of the p53 antibody titer and the response to therapy suggests that p53 antibodies could represent a useful tool for checking the response to therapy and for monitoring some relapses before they are clinically detectable.

Introduction

In 1979, DeLeo et al. (1) showed that the humoral response of mice to some methylcholanthrene-induced tumor cells was directed to the p53 protein. It was later found that animals bearing several types of tumors elicited an immune response specific for p53 (2–4). In 1982, Crawford et al. (5) first described p53-Abs in 9% of breast cancer patient sera. Caron de Fromentel et al. (6) later found that such antibodies were present in sera of children with a wide variety of cancers. The average frequency was 12%, but this figure increased to 20% in Burkitt lymphoma.

Those studies, performed in the early 1980s, were virtually ignored for more than 10 years due to the lack of interest in p53 during that period. In the early 1990s, it was discovered that the p53 gene is the most common target for molecular alteration in every type of human cancer. Alteration of the p53 gene occurs predominantly by missense mutations occurring nonrandomly throughout the central region of the protein, thereby inactivating its DNA binding properties (7). The wild-type p53 protein is regulated by rapid turnover, and the level present in the nuclei of normal cells is below the sensitivity of immunohistochemical analysis detection systems. In tumoral cells, changes in the p53 conformation and/or cellular environment lead to metabolic stabilization of the mutant p53 and its accumulation in the nuclei (8, 9). Several analyses have shown that there is a close correlation between accumulation of p53 protein and mutation of the p53 gene (10). These findings have shed new light on the presence of serum p53-Abs. More recent works have indicated that anti-p53 antibodies can be found in most human cancers (11–15). There is generally a good correlation between their frequency and that of p53 gene alterations (16, 17). Several multifactorial studies showed a very good correlation between the presence of p53-Abs, accumulation of the mutant protein in the tumor, and the presence of a mutation in the gene (13, 18, 19).

Detailed analysis of these antibodies indicates that accumulation of the p53 protein in tumor cells is responsible for the appearance of autoantibodies with epitopes directed toward the immunodominant region of the p53 protein (12, 16, 20, 21). Although the detection of p53-Abs cannot replace molecular or immunohistochemical analysis of p53 alteration, it has some specific applications. First, it is now well documented that p53-Abs can be detected in patients with a high risk of cancer several years before the clinical manifestation of the disease (22, 23). A second application, which has not been well documented thus far, concerns the use of p53-Abs for follow-up of patients during their treatment (15, 24).
In the present study, from an original group of 167 prospectively studied patients, we describe the results of a 25-month follow-up of 32 patients. Of these patients, 16 were seropositive for p53 antibodies and showed a good correlation between evolution of the p53-Ab titer and response to therapy. Control analysis demonstrated the specificity of the p53-Abs variation.

**Patients and Methods**

**Patients.** The initial cohort included 167 consecutive patients with histologically proven lung cancer during a 25-month period from April 1993 to May 1995 in three university hospital in Paris, France (Saint-Louis, Saint-Antoine, and Tenon hospitals). The primary goal of this prospective study was to evaluate the prognostic value of p53 antibodies in lung cancer. This protocol has been approved by the Consultative Committee for the Protection of Persons in Biomedical Research. SCLC patients $(n = 91)$ were treated in these three institutions with equivalent therapeutic regimens: six cycles of platinum and etoposide-based CT. After the second course of CT, additional concomitant mediastinal radiotherapy was proposed to patients with localized disease (patients with tumors confined to one hemithorax). In these patients with limited disease $(n = 50)$, prophylactic cranial irradiation was proposed to clinically complete responders. Only NSCLC patients $(n = 76)$ from Saint-Louis Hospital were included in the study and monitored for p53 antibodies because of the heterogeneity of treatments proposed to NSCLC patients in the three participating centers. The characteristics of the whole cohort of patients and the results of the prognostic impact study will be described elsewhere.5

Among this group of patients, 16 fulfilled the following eligibility criteria: (a) they were inoperable; (b) they were treated by CT alone or by chemoradiotherapy; (c) they were p53 seropositive; (d) they had a sufficient number of p53 antibody determinations during CT (i.e., at least three serum samples, with a survival of at least 4 months); and (e) they had evaluable lesions. Among p53 seronegative patients, 16 were selected for study and matched to the p53-seropositive patients on the basis of their response to therapy and their survival status.

Survival was evaluated from the date of histological diagnosis to the date of death or of the last follow-up visit. Response was assessed according to Eastern Cooperative Oncology Group standard guidelines (25). Pathological diagnosis was obtained mainly from bronchial biopsy samples using WHO histological criteria (26).

Sera were obtained after diagnosis, but prior to any treatment; then at each follow-up evaluation visit (every 1–3 months), 7 ml of whole blood were centrifuged at 3000 rpm for 15 min, and the supernatant was stored at $\pm 80^\circ$C until use. All analyses were done in duplicate. Sera were analyzed with the observer blind to patient status.

Survival data were updated in October 1997. Overall survival was analyzed using the Kaplan-Meier method. Curves were compared with the log rank test using the statistical package Statview 4.02 (Abacus Concepts, Berkeley, CA). The association between a 50% decrease in the p53-Ab titer and partial or complete response was tested by Fischer’s exact due to the small sample size.

**ELISA for p53 Antibodies.** ELISA and its characteristics have been fully described elsewhere (17). We devised a highly specific ELISA by testing all sera with two antigen preparations; the first contained the relevant antigen, p53, whereas in the second preparation, this antigen was omitted. Polystyrene flat-bottomed microtiter plates (F16 Maxisorp, Nunc) were coated with either p53 or control extract in carbonate buffer. Plates were dried for 24 h at 37°C. After saturation, they were 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 containing 0.05% Tween 20; 100 μl of blocking buffer (PBS 0.2% Tween 20, 5.0% dried nonfat milk) were added per well. After 1 h at 37°C, the wells were washed as described above. Then, 100 μl of sera (diluted 1:100 in PBS, 5.0% dried nonfat 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 with 0.05% Tween 20, 100 μl of an antihuman IgG peroxidase conjugate diluted 1:8 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 because we had demonstrated that all these p53-Abs include IgG, we used an antihuman IgG antibody. Plates were then washed five times and developed using a 3,3′,5′,5′-tetramethylbenzidine substrate. The reaction was stopped after 10 min by adding 1 M sulfuric acid. Plates were then read at 450 nm using a MR 5000 ELISA reader (Dynatech Laboratories). All of these manipulations were performed simultaneously on a plate coated either with p53 protein as described above or with the control extract. The sensitivity and the specificity of this assay have already been described (17). For the purpose of routine screening, each serum was diluted 1:100. For the quantitative measurement of p53-Abs in positive patients during their follow-up, sera were diluted such that there was no saturation during color development. For a given patient, the same dilution was used for each sample, and all samples were processed in the same experiment. Most of these sera were also tested by immunoprecipitation as described previously (17).

**Peptide ELISA for p53 Antibodies.** The major sites of recognition by p53-Abs are localized in the amino and carboxyl termini of the protein. This has been repeatedly demonstrated by testing the binding of sera against a series of 77 peptides overlapping the complete p53 protein. The majority (98%) of positive sera reacted with an amino-terminal peptide, and 48% reacted with a carboxyl-terminal peptide (20). We then devised a new ELISA with six peptides (18). Five peptides correspond to p53 peptides: peptide 1, EPPLSQEDSLWKLLPENNVLSPL, corresponds to the first immunodominant epitope localized in the amino terminus; peptide 2, DDLMLSPDDIEQWFT, and peptide 3, SPDDIEQWFTEDPG, correspond to the second immunodominant epitope localized in the amino terminus; and peptide 4, EALELKDAAQAGKEPGGSRAHSHSLK, and peptide 5, GSRAHSSHLKSKKQSTSRHKKLMF, correspond to two immunodominant peptides localized in the carboxyl termi-
Fig. 1 Variations in p53 antibodies. For all but two patients (GAT and BRE), p53 antibodies were monitored using either entire p53 (left row) or with synthetic peptides (right row). For ELISA using the entire p53, results are expressed as the ratio between p53 and the control antigen as described in “Patients and Methods.” For ELISA using peptides, absorbance is given for each assay. Values of the control peptide have been deduced for each measurement.
Table 1 Characteristics of the patients studied

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<th>Patient</th>
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<th>Therapy</th>
<th>Response</th>
<th>Status at last follow-up</th>
<th>Survival (days)</th>
<th>Cause of death</th>
<th>p53-Ab</th>
<th>Variation of p53-Ab*</th>
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* Three levels of variation of p53-Abs are shown: -, no decrease; +, decrease of less than 50%; ++, decrease of more than 80% or disappearance.

LCC, large cell carcinoma; RT, radiotherapy; CR, complete response; NR, no response; PR, partial response.

nus of p53. Peptide 6 was a human serum albumin peptide used as an internal negative control in every experiment. This assay was conducted as described previously with streptavidine-coated plates, biotin-labeled peptides, and direct binding of human antibodies diluted 1:100 for assay (18). Results were expressed as a ratio between binding to p53 peptides and control.

HAV ELISA. The detection of total antibodies to HAV was performed with a commercially available ELISA (HAV Total, Sanofi Diagnostic Pasteur, Paris, France). The assay was performed according to the manufacturer’s instructions and had the following specifications: 1:5 to 1:100 diluted patient serum and peroxidase-conjugated anti-HAV antibodies were added for 4 h at 40°C to microplate wells coated with HAV antigen. If any anti-HAV antibodies were present in the patient serum, this resulted in competition with the conjugate. After washing, the substrate 3,5,3,5-tetramethylbenzidine solution was added for 30 min at room temperature. The enzymatic reaction was stopped by adding 1 N sulfuric acid. The absorbance was measured at 450 nm on a spectrophotometer (Dynatech MR5000). A
p53 Antibodies in Sera of Patients with Lung Cancer.

In 1993, we set up a prospective study in the respiratory departments of the Saint Louis, Saint-Antoine, and Tenon Hospitals. p53 antibodies were evaluated in the sera of every patient with diagnosed lung cancer. The results of that study will be published elsewhere. In the present work, 32 patients were eligible for detailed analysis. Sixteen patients had p53-Abs at the time of diagnosis (7 NSCL and 9 SCLC), whereas 16 others were negative (5 NSCLC and 11 SCLC). All these patients had a follow-up of at least 6 months, with 4–10 sera taken during therapy.

Monitoring of p53 Antibodies during Follow-up of Treated Lung Cancer Patients. The level of p53-Abs was monitored during treatment of the various patients. The use of an internal control in ELISA led to a quantitative assay as shown in Fig. 1 and summarized in Table 1. Of the 16 patients analyzed here, 12 had a substantial decrease in p53-Abs (over 50% compared to their initial level), and 4 had no change in the level of p53-Abs, or else had a decrease of less than 50% (RAM, shown in Fig. 1). An internal control in ELISA led to a quantitative assay as shown in Fig. 1 and summarized in Table 1.

Fig. 2 Monitoring of p53 antibodies using immunoprecipitation of in vitro translated p53. +, positive control (HR231 monoclonal antibody); −, negative control (sera from a blood donor); M, molecular weight markers. The second band below p53 corresponds to a truncated p53 produced during in vitro translation.

These observations indicate that the level of p53-Ab decreases during the therapy but do not give any information concerning the specificity of this variation.

The Variation in p53 Antibodies Is Specific. Most of these patients received CT as part of their treatment. It is widely known that such treatment can eventually lead to partial immunosuppression or other immunological disorders that can induce nonspecific disappearance of immunoglobulins. The level of total serum immunoglobulins did not change significantly during the course of the follow-up (data not shown). Nevertheless, we cannot exclude the possibility that there was a general loss of circulating antibodies. To control that only p53-Abs varied during the course of therapy, we checked the humoral response directed toward another antigen not related to neoplasia. We chose to test HAV antibodies that are frequently present in the sera of a normal population. In our series, all patients were found to carry HAV antibodies at the time of diagnosis and were thus suitable for this follow-up analysis. Fig. 3 clearly shows that the level of HAV antibodies did not change during the course of therapy, whatever the evolution of p53-Abs. For each patient, the level of variation in HAV antibodies never attained more than 15% (Fig. 3 and data not shown). Similar studies in patients without any p53-Abs showed similar results (Fig. 3 and data not shown).

Variation in p53 Antibodies Specific for Various Epitopes. We previously showed that these antibodies recognized specific immunodominant epitopes localized in the amino and carboxyl termini of human p53 (20). Using the peptide ELISA described in “Patients and Methods,” we performed a detailed analysis of the epitopes recognized by the serum p53-Abs and their variation during therapy. Five sera were specific for the amino terminus, whereas seven sera also recognized the carboxyl terminus of p53. No serum was specific for the carboxyl terminus of p53 as we had previously demonstrated. None of the sera showed any cross-reactions with the control peptide. Analysis of the behavior of p53-Abs during therapy is shown in Fig. 1. For all sera, variations in the different subpopulations of immunoglobulins directed toward various epitopes of the protein were strictly identical to those detected using the entire p53 protein (Fig. 3 and data not shown). Furthermore, comparison of the variation in p53-Abs with the two ELISAs emphasized that true p53-Abs are detected by both ELISAs.
Detection of p53 Antibodies by Immunoprecipitation. It has been suggested that ELISA can lead to false positive results due to cross-reactions with other proteins that may be present in the cell extract. Although the use of a control extract should alleviate such false-positive results, we controlled the presence of p53-Abs by immunoprecipitation of native p53 obtained by in vitro transcription and translation. In this assay, p53 was the only labeled protein. All sera except one (BEA) that were positive by ELISA could also immunoprecipitate p53 (Fig. 2 and data not shown). The remaining serum was repeatedly found to be weakly positive for the ELISA procedure, showing the higher sensitivity of ELISA compared to immunoprecipitation. None of the sera that were negative by ELISA could bring down p53 in this immunoprecipitation assay. Such an assay could also be quantitative, as shown in Fig. 2. The variation in immunoprecipitated p53 on the gel was similar to the signal shown in Fig. 1 by ELISA.

All results described here indicate that the variation in p53-Abs detected in sera of patients was indeed specific.

p53 Antibody Variations and Clinical Response to Therapy. A decrease in p53-Ab was observed in 12 patients during therapy. There was a trend toward an association between a decrease in p53-Ab titers and overall response to treatment, i.e., partial or complete response (P = 0.0571; Fischer’s exact test). When survival curves of patients with a 50% decrease in their p53-Ab titers and of patients without a significant decrease in these titers were plotted, there was a trend toward an association between better survival and decreased p53-Ab titers of more than 50%. However, the difference of survival between the two groups did not reach statistical significance (P = 0.163), presumably because of the small sample size. All three patients still alive at the time of updating experienced a substantial decrease in their p53-Ab titers, with two of them becoming p53 seronegative (BEL and PEC).

Discussion

The first description of p53-Abs in 1984 was not fully understood due to the lack of knowledge on the p53 mutation and its role in human cancers (5). Since 1991, a body of evidence has demonstrated that these p53-Abs are linked to the presence of p53 accumulation in human tumors (27). p53-Abs are not present in the normal population, and the frequency of cancer patients with such Abs is proportional to the frequency of p53 alterations (17). Although most patients with p53-Abs harbor a p53 mutation, the opposite is not true, and recent multifactorial analysis indicates that only 30% of patients with p53 alterations developed a humoral response toward p53.

In the present study, we address the question of the fate of these p53-Abs during therapy. Thus far, only a few works have dealt with this question (15, 24). In the present prospective study, 32 lung cancer patients were chosen for a detailed analysis of the behavior of p53-Abs during therapy. Of the 16 patients without p53-Abs at the time of diagnosis, none underwent a p53 humoral response during treatment, whatever the fate of the disease. This observation has been extended to more than 100 other patients with various types of cancer. These results confirm the observation that each patient might have an inherent capacity to develop such p53 humoral response. Such behavior could be linked to the position of the mutation in the p53 protein, as previously suggested (13), and/or to some specific genetic background. Indeed, immunodominant p53 epitopes might need specific histocompatibility complex class I molecules to be properly presented to the immune system and therefore lead to the development of p53 antibodies.

A decrease in p53-Abs was observed in 12 patients during therapy. The finding that there is a true disappearance of p53-Abs is emphasized by the observation that such a decrease can be seen both on the whole native p53 by two immunological techniques and with various peptides corresponding to different parts of the p53 protein. Nevertheless, it was essential to show that such a decrease of p53-Abs from sera is specific because the overall serum antibodies level could be affected during therapy.

Fig. 1 Variations in anti-p53 and anti-HAV antibodies during follow-up of four patients (FRO, BEN, VAN, and RAM). The percentage of anti-p53 and anti-HAV antibodies is expressed as a function of elapsing time (t). For the sake of comparison, the level of anti-p53 and anti-HAV antibodies at the time of diagnosis (t₀) was considered to be 100%.
This was demonstrated by examining the level of another humoral response. HAV was chosen because it is known that more than 90% of the population over 60 years old has such antibodies. There was no variation in these antibodies in any patients whether they were positive or negative for p53-Abs, thus confirming the specificity of p53-Ab variation. Furthermore, such a correlation with the p53-Ab titer and tumor growth is well demonstrated in patient BEL. p53-Abs were detected in this patient 2 years before the detection of the tumor. During those 2 years, this patient was carefully monitored, but no treatment was administered because there was no clue at that time that p53-Ab could be an early indicator of cancer. During the 2-year period, the level of p53-Abs increased concomitantly with the development of the tumor that was clinically detected 2 years after the initial detection of p53-Abs. The decrease in p53-Abs was only seen after the beginning of treatment. There was no variation in HAV antibodies during the entire follow-up of this patient before or after the detection of the tumor (data not shown). The behavior of the tumor in this patient indicates that p53-Abs are linked to the presence of tumor growth. This finding raises interesting questions concerning the immunogenicity of the p53 protein. We previously demonstrated that the p53 protein is highly immunogenic, with a specific humoral response directed toward immunodominant epitopes localized in the amino and carboxyl termini of the protein (12, 16, 21). The present work shows that high levels of p53-Abs require the presence of tumoral cells; otherwise, there is a very rapid decrease in these antibodies during and after therapy. In some patients, this decrease corresponds to the half-life of human serum IgG, suggesting that continuous exposure to p53 is necessary to maintain a steady-state level of p53-Abs.

Twelve of 16 patients with p53-Abs had a decrease in the p53-Ab level of more than 50% compared to the initial titer. Among these patients, eight underwent a complete response to therapy, whereas four obtained only a partial response. Of the five patients without any variation in their p53-Ab levels, two showed a partial response, whereas no response was observed in the three others. Although the number of patients was too small for statistical analysis, no patient with a complete response had a stable level of p53-Abs, whereas patients with no response always maintained an unvarying level of p53.

One of the benefits of using p53-Abs for follow-up of patients could be the detection of the reappearance of such antibodies after therapy. In lung cancer, the survival of patients is usually rather short and can preclude such studies. In this study, three complete responder patients relapsed without any rise in their p53 antibody titers (BEA, BEN, and DEJ). Two of these patients had cerebral metastasis as the unique site of relapse. The remaining patient had an initial weakly positive ELISA test and died only 6 weeks after relapse was diagnosed, a period presumably too brief to elicite a detectable p53 humoral response. Brain was long considered as an immune “sanctuary.” For instance, no p53 antibody was detected in a series of patients with tumors of the central nervous system, whereas p53 mutations were actually detected in tumoral tissue (28). An alternative explanation would be that relapse was due to a tumoral clone retaining metastasizing or invasive potential but without any p53 mutation. In a recent work, we showed that temporal changes in the level of p53-Abs could be closely correlated with disease progression or regression in colon cancer, where a longer follow-up is possible (24). In breast cancer, we were able to detect the reappearance of p53-Abs 2 years after the initial therapy. This increase in p53-Ab was detected 3 months before the detection of a relapse.7 Thus, in such tumor types, monitoring p53-Ab positive patients revealed a potential for evaluating response to CT/radiotherapy or detecting intraclinical relapse. p53-Ab could be a useful tool for oncologists in their attempt to analyze the patient response to therapy in addition with standard clinical and radiological evaluation.

Acknowledgments

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References


cancer patients appears to be dependent on the type of p53 mutation.

G Zalcman, B Schlichtholz, J Trédaniel, et al.