Circulating Antibodies against p53 Protein in Patients with Ovarian Carcinoma

Correlation with Clinicopathologic Features and Survival

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BACKGROUND. Genetic alterations of the p53 tumor suppressor protein are the most frequent molecular events in human carcinogenesis. For as yet unknown reasons, mutant p53 often acts as an immunogen for autoantibody generation. These autoantibodies can be detected in the serum of cancer patients. The presence of such antibodies has been identified in a subset of patients with ovarian carcinoma, but their clinical significance has not been investigated.

METHODS. Serum samples from patients with ovarian carcinoma were quantitatively analyzed for the presence of p53 autoantibodies with a time-resolved immunofluorometric procedure. Tumor p53 overexpression was assessed by immunohistochemical analysis of tissue sections. Kaplan–Meier survival curves were calculated for p53 antibody positive and negative patients, and the Cox model was used to evaluate the strength of the associations between the presence of serum p53 antibodies and cancer relapse or death, and also between the presence of such antibodies and other clinicopathologic features.

RESULTS. p53 antibodies were detected in the serum of 41 of 174 patients with ovarian carcinoma (24%). Antibody levels ranged from a few hundred to 9×10^6 arbitrary Units/L, and fluctuated during the course of the disease. p53 antibody positive patients tended to have tumors overexpressing p53, but the association between the two parameters was not statistically significant ($P = 0.13$). There was also no association between the presence of p53 antibodies and clinical stage, tumor histologic type, or overall patient survival. However, these antibodies were more frequently present in patients older than 50 years ($P = 0.001$), in patients with moderately or poorly differentiated tumors ($P = 0.001$), and in patients who received chemotherapy ($P = 0.015$), and who suffered relapse after surgery ($P = 0.018$). In univariate analysis, p53 antibody positive patients were at an increased risk for relapse but not death. In multivariate analysis, the differences in disease free and overall survival between patients who were p53 antibody positive or negative were not statistically significant.

CONCLUSIONS. p53 autoantibodies are found frequently in the serum of patients with ovarian carcinoma. The presence of such autoantibodies was associated with older patient age, more aggressive tumors, and reduced patient disease free survival. In multivariate analysis the prognostic value of p53 autoantibodies was not statistically significant. ***Cancer* 1996; 77:2146–52.** © *1996 American Cancer Society.*

KEYWORDS: p53 protein, p53 autoantibodies, ovarian carcinoma, tumor suppressor genes, cancer monitoring, tumor markers.

Inactivation of the p53 tumor suppressor gene is the most common genetic alteration in human neoplasia.[1] The genetic lesions most often identified are missense point mutations in evolutionarily conserved regions of the p53 gene, usually accompanied by loss of the

corresponding wild type allele. It is generally accepted that point mutations result in modification of the conformation and increase of the stability of the protein. Increased stability leads to accumulation of mutant p53 in tumor cells, and the mutant protein can be detected readily by immunohistochemical or immunologic methods. In contrast, the wild type p53 protein is expressed at very low levels and is usually undetectable.

The mutant p53 protein has not been detected in the serum of cancer patients.[2] This precludes the utility of this molecule as a marker for disease diagnosis and patient monitoring, even in cancers with high p53 mutability (e.g., ovarian, colon, and breast). p53-specific autoantibodies have been found in the serum of patients with various neoplasms. From the wide variety of cancers studied so far, p53 antibodies are most commonly found in tumors of the breast (9–26%),[3–6] lung (13–24%),[7,8] colon (16–25%),[9–11] and ovaries (15–29%).[9,10,12]

Although the immunogenicity of mutant p53 and its clinical implications have recently attracted attention, the molecular events leading to this immune response have not as yet been adequately explained. The most widely accepted hypothesis is that overexpressed mutant p53 is considered a nonself protein, triggering the immunocompetent cells for the secretion of anti-p53 antibodies in a proportion of cancer patients. It has also been suggested that the development of p53 antibodies is dependent on complexing of the mutant protein with members of the heat shock protein 70 family (HSP70).[4] Winter et al.[7] showed that the induction of antibodies in lung cancer patients is dependent on the type of the p53 gene mutation. The same group also proposed that p53 antibodies are found in the serum only when p53 accumulation is detected in the tumor cells, but gene mutation or protein overexpression does not automatically lead to immune response.[7] More recently, it has been demonstrated that p53 antibodies are directed toward immunodominant epitopes localized in the amino and carboxy terminal end of the protein.[5,8,13]

The p53 immune response and its correlation with clinicopathologic parameters have been studied for breast and colon cancer patients. p53 antibodies were shown to associate with aggressiveness and poor outcome in patients with breast cancer (high histologic grade, absence of steroid hormone receptors, history of second primary cancer).[5,6,10,14] In colon cancer, p53 antibodies correlated with histologic differentiation grade, tumor shape, and tumor invasion into blood vessels.[11] The correlation of p53 antibodies to clinical outcome in ovarian carcinoma has not yet been investigated.

Ovarian carcinoma is currently the leading cause of death due to gynecologic malignancy in the United States.[15] Although the underlying molecular mechanisms of ovarian carcinoma are still poorly understood, it is believed that activation of protooncogenes and loss of critical tumor suppressor genes may play an integral role in early ovarian oncogenesis. Allelic losses and mutations of the p53 gene are considered key genetic alterations for the development of this carcinoma.[16] It has been found that 50% of epithelial ovarian carcinomas overexpress the p53 protein.[17,18] Recently, our group and others have shown that ovarian tumors frequently elicit an immune response for the production of p53 autoantibodies.[10,12] These antibodies can be detected in the serum and in the ascites fluid that usually accompanies the tumor (Angelopoulou K, Diamandis EP, 1996).[10]

In this article, we examine the association between p53 antibodies and other clinicopathologic features, as well as their association with disease free and overall survival, in a group of 174 patients with primary ovarian carcinoma.

MATERIALS AND METHODS

Study Population

One hundred and seventy four women (age range, 14–88 years; median, 56) with primary epithelial ovarian carcinoma were included in this study. All patients were treated and underwent follow-up at the Toronto Hospital, Toronto, Canada. Follow-up time of cancer patients ranged from 1 to 174 months, with a median of 34 months. Serum samples from all patients were collected over a 12-month period and stored at −70°C until analysis. In all patients, sera were collected within 6 months from diagnosis. Previous work has shown that p53 antibodies are stable for at least 3 years if stored at −70°C. For 30 patients, formalin-fixed, paraffin-embedded tissue was also available for immunohistochemical analysis.

Cell Line

The cell line COLO 320 HSR(+) was used as a source of mutant p53 protein and was obtained from the American Type Culture Collection (Rockville, MD). This is a colorectal carcinoma cell line that has been shown to express high levels of p53 protein as a result of a mutation at codon 248 of the p53 gene.[2,19] Cells were grown in suspension in RPMI culture medium containing 10% fetal calf serum and antibiotics.

Antibodies

The mouse monoclonal anti-p53 antibody PAb240, which recognizes an epitope on 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 by Dr. D. Lane, University of Dundee, Scotland. The human anti-p53 antibodies in the serum were detected with a goat antihuman immunoglobulin G (IgG) antibody conjugated to alkaline phosphatase (Jackson Immunoreasearch, West Grove, PA). The mouse monoclonal anti-p53 antibody DO-7 was used for immunohistochemistry. This antibody recognizes an epitope that resides between amino acids 35 and 45 of the p53 protein and reacts with both wild type and mutant forms of the protein.[20]

Immunoassay

For the quantitative analysis of p53 antibodies in serum, we used a time-resolved immunofluorometric technique that has been described in detail elsewhere.[9,21] The method is based on the measurement of alkaline phosphatase activity with a highly sensitive fluorogenic substrate as described elsewhere.[22,23]

p53 Antibody Positivity and Quantification

An arbitrary system was used to calibrate the immunoassay as follows. A highly positive serum was assigned a 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.

Immunohistochemistry

Four-micron sections 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. The slides were placed in a thermoresistant plastic container filled with 10 mmol/L citrate buffer, pH 6.0, and processed in a microwave oven 5 times for 5 minutes each at 750 W. The sections were allowed to cool in the container at room temperature for approximately 20 minutes and then rinsed in Tris-buffered saline (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 minutes and incubated with the primary antibody (DO-7) at 37°C for 1 hour. The slides were then washed in TBS two times and incubated with the secondary antibody (biotinylated goat antimouse antibody) at 37°C for 45 minutes. They were washed again two times in TBS and incubated at 37°C for 45 minutes with the avidin-peroxidase conjugate. The biotinylated antibody, avidin-HRP, and substrate were components of the Vectastein ABC kit commercially available from Vector Laboratories (Burlingame, CA). After another washing with TBS, the slides were incubated for 4 minutes at room temperature with diaminobenzidine (DAB) solution (0.5% DAB in TBS, pH 7.6). After a final washing with water, the slides were counterstained with hematoxylin for 2 minutes, dehydrated, cleared, and mounted.

Statistical Analysis

For survival analysis, two different end points of follow-up—cancer relapse (either local recurrence or distant metastasis) and death—were used to calculate disease free and overall survival, respectively. Disease free survival was defined as the time interval between surgery and recurrence. Overall survival was defined as the time interval between surgery and death. The associations between p53 antibodies and other prognostic markers were examined with the chi-square test. The Cox proportional hazards regression model was used to evaluate the strength of the associations between presence of p53 antibodies and disease relapse or death. This analysis was conducted at both univariate and multivariate levels. Kaplan–Meier survival curves were constructed for the p53 antibody positive and p53 antibody negative patients. The log rank test was used to examine the differences between the Kaplan–Meier curves. Computer software SAS (SAS Institute, Cary, NC) and EGRET (Statistics and Epidemiology Research, Seattle, WA) were used for data analyses. Differences were considered significant when the probability values obtained from the statistical tests were 0.05 or less.

RESULTS

p53 Antibodies in the Sera of Patients with Ovarian Carcinoma

Serum samples from 174 patients with ovarian carcinoma were analyzed for the presence of p53 autoantibodies. Antibodies were detected in 41 of 174 patients (24%). p53 antibody levels were measured in all positive sera using an arbitrary quantification system (Fig. 1). The antibody titers varied from a few hundred (e.g., Patients 15 and 23) to 9,000,000 arbitrary U/L (Patient 8). Twelve of 41 positive patients had p53 antibody concentrations higher than 10,000 U/L. For 26 positive patients, multiple sera were obtained at different times during the course of the disease. All samples from the same patient consistently scored positive, but the concentrations varied significantly in some cases. For example, a 15-fold difference was found between two sera from Patient 16 (Fig. 1).

For Patients 4, 18, 19, and 39 (who had at least six sequential serum samples each), we measured p53 autoantibodies and the tumor marker CA-125. In general, parallel changes for the two serum analytes were

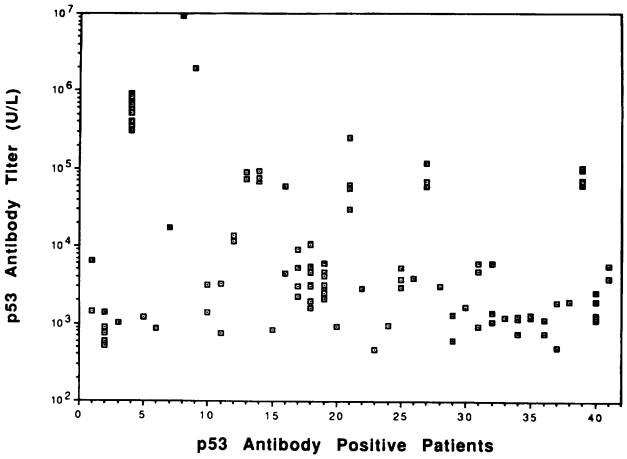

FIGURE 1. p53 antibody concentrations quantified in the serum of 41 p53 antibody positive ovarian carcinoma patients. Many patients have multiple samples obtained during the course of their disease.

observed, suggesting that p53 antibody titers, like CA-125, correlate with tumor volume (data not shown).

Association between the Presence of p53 Antibodies in the Serum and Expression of p53 in the Corresponding Tumor

In a subset of 30 patients (of whom 16 were p53 antibody positive and 14 were p53 antibody negative), p53 expression of the tumor was immunohistochemically evaluated with the DO-7 antibody. The results are presented in Table 1. Among 13 tumors that were p53 antigen positive, 9 (69%) were associated with p53 antibody positive sera; among 17 tumors that were p53 antigen negative, 7 (41%) were associated with p53 antibody positive sera. Although there was a preference for p53 antigen positive tumors to be associated with p53 antibody positive sera, the difference did not reach statistical significance ($P = 0.13$).

Association between Presence of p53 Antibodies and Clinicopathologic Features

The relationships between the p53 antibodies and tumor clinicopathologic features were examined and the results are presented in Table 2. The presence of p53 antibodies was significantly associated with older patient age. The p53 antibody positivity rate in patients younger than 50 years was 9%; in patients 50 years and older, the positivity was 33% ($P = 0.001$). No significant association was observed between the presence of antibodies and clinical stage. Serum p53 antibodies were present with similar frequencies in patients with early (Stage I/II) or late (Stage III/IV) tumor stage (20% and 28%, respectively; $P = 0.34$). The presence of p53 antibodies was significantly associated with the degree of tumor cell differentiation. Among the p53 antibody positive patients, 6% had well differentiated tumors (Grade 1), 38% had moderately differentiated tumors (Grade 2), and 28% had poorly differentiated tumors (Grade 3) ($P = 0.001$). In terms of histology, tumors were classified as serous (68 patients) and others (39 patients). Patients with serous carcinomas tended to be p53 antibody positive more frequently, but the difference was of borderline statistical significance ($P = 0.074$). The presence of p53 antibodies was also significantly associated with higher frequency of patient relapse ($P = 0.018$) but not with patient overall survival ($P = 0.34$). Kaplan–Meier survival curves (Fig. 2), showed that p53 antibody positive patients had significantly shorter disease free survival than the p53

TABLE 1
Association Between the Presence of p53 Antibodies in Serum and p53 Antigen in the Corresponding Tumor

Patient number[a]	p53 Antibodies in serum, U/L[b]	p53 Antigen in tumor[c]
2	515–1,412	+++
4	300,000–922,400	+++
8	9,347,167	+++
10	1,387; 3,195	+++
12	11,586; 13,786	++
16	4,475; 59,330	++
22	2,882	++
24	963	+
25	2,992–5,247	+
26	3,825	–
27	59,665–116,222	–
29	618; 1,295	–
31	920; 4,722	–
32	1,047–6,015	–
34	740–1,262	–
37	492; 1,892	–
42	–	+++
43	–	+++
44	–	+
45	–	+
46	–	–
47	–	–
48	–	–
49	–	–
50	–	–
51	–	–
52	–	–
53	–	–
54	–	–
55	–	–

[a] The code number of each patient is the same as in Figure 1.
[b] (–): p53 antibody- or p53 antigen-negative sera or tumors, respectively. For patients with more than two sera tested, the range of values is given.
[c] Semiquantitative assessment with a scale from weak (+), moderate (++), or strong positivity (+++).

TABLE 2
Associations between p53 Antibody Status and Clinical and Pathological Features of Ovarian Cancer

Feature	Number of patients (%) p53 Ab (−)	p53 Ab (+)	*P*
Age (yr):			
<50	52 (91.2%)	5 (8.8%)	
≥50	71 (67.3%)	35 (32.7%)	0.001
Stage:			
I–II	31 (79.5%)	8 (20.5%)	
III–IV	73 (71.6%)	29 (28.4%)	0.34
Grade:			
I	48 (94.1%)	3 (5.9%)	
II	24 (61.5%)	15 (38.5%)	
III	39 (72.2%)	15 (27.8%)	0.001
Histology:			
Serous	68 (73.1%)	25 (26.9%)	
Others	39 (86.7%)	6 (13.3%)	0.074
Chemotherapy:			
Yes	83 (71.6%)	33 (28.5%)	
No	42 (89.4%)	5 (10.6%)	0.015
Relapse:			
No	55 (84.6%)	10 (15.4%)	
Yes	59 (67.8%)	28 (32.2%)	0.018
Death:			
No	86 (78.2%)	24 (21.8%)	
Yes	43 (71.7%)	17 (28.3%)	0.34

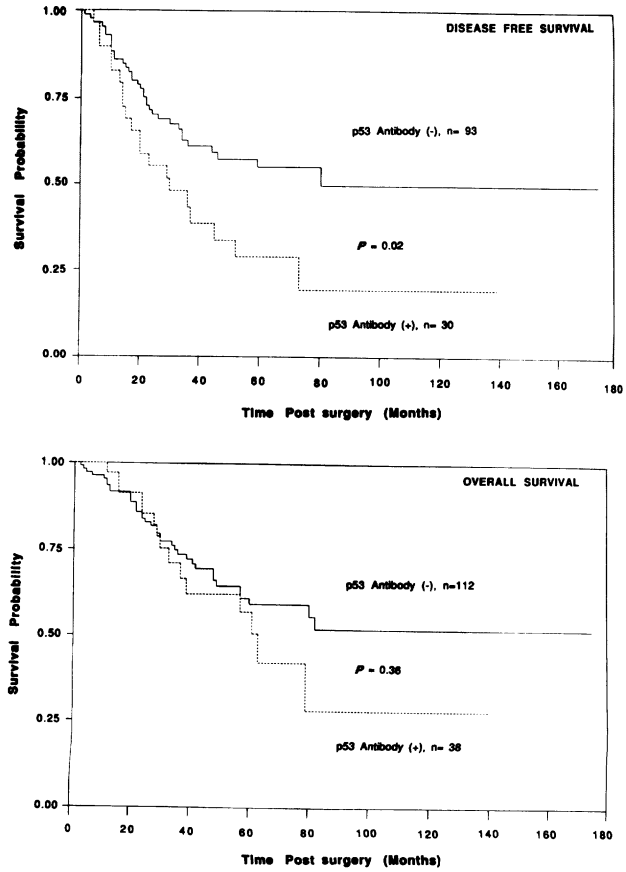

FIGURE 2. Kaplan–Meier survival analysis of disease free (upper panel) and overall survival (lower panel) of ovarian carcinoma patients who were either positive or negative for serum p53 antibodies.

antibody negative patients ($P = 0.02$). No significant association with overall patient survival was found ($P = 0.36$).

The strength of the associations between p53 antibodies and disease free or overall survival is shown in Table 3. The presence of p53 antibodies was associated with cancer relapse but not death in univariate analysis. When the other prognostic factors were included in the Cox model (multivariate analysis), the presence of p53 antibodies was not significantly associated with reduced disease free or overall survival.

DISCUSSION

Analysis of sera from 174 women with ovarian carcinoma revealed that the immune response against p53 is a common event in ovarian carcinoma. Twenty-four percent of patients had serum p53 antibodies, the concentrations of which ranged dramatically from a few hundred to 9,000,000 arbitrary U/L. Follow-up of some patients showed that p53 antibody levels roughly parallel changes of CA-125 concentrations. This observation is consistent with previously published data, which suggested that the p53 antibody test may have value for patient monitoring during therapy.[10] These data, however, should be considered as preliminary because they are based on a small patient population.

TABLE 3
Associations Between Serum p53 Antibodies and Patient Survival

p53 Ab status	OR[a]	95% CI[b]	*P*
Univariate analysis:			
Disease-free survival[c]			
p53 Ab (−)	1.00		
p53 Ab (+)	1.98	1.14–3.43	0.02
Overall survival[d]			
p53 Ab (−)	1.00		
p53 Ab (+)	1.33	0.73–2.44	0.35
Multivariate analysis[e]:			
Disease-free survival[f]			
p53 Ab (−)	1.00		
p53 Ab (+)	0.69	0.32–1.51	0.35
Overall survival[g]			
p53 Ab (−)	1.00		
p53 Ab (+)	0.61	0.27–1.38	0.23

[a] Odds Ratio. [b]Confidence interval. [c]116 patients. [d]150 patients. [e]Adjusted for age, clinical stage, histological grade and type, and chemotherapy. [f]74 patients. [g]98 patients.

Future studies on this issue are clearly necessary. In our study, multiple sera were tested over many months, during tumor progression or regression and therapeutic manipulations (chemotherapy, surgery). None of the patients whose serum originally scored positive was converted to negative, despite changes in titers. One of the possible interpretations of this finding is that p53 autoantibodies are long lived and circulate in blood for many months or years.

p53 autoantibodies were detected with higher frequency in older patients (older than 50 years) and in patients with high histologic grade tumor who were treated with chemotherapy after surgery. In general, patients who receive chemotherapy have more advanced disease than patients who do not receive chemotherapy. Patients with p53 antibodies more frequently had tumors of serous histology, but the association was of borderline significance ($P = 0.074$). No association was found between the presence of autoantibodies and the clinical stage of disease. Furthermore, presence of p53 antibodies was associated with more frequent cancer relapse ($P = 0.018$) but not with death. Kaplan–Meier survival curves revealed that p53 antibody positive patients had significantly shorter disease free survival than p53 antibody negative patients. The overall survival, however, did not differ significantly between the two groups (Fig. 2). In multivariate analysis, the presence of p53 antibodies was not significantly associated with either disease free or overall survival. These data support the view that the presence of p53 autoantibodies is associated with other parameters characteristic of aggressive tumors and that these antibodies have no independent value for patient prognosis.

The molecular mechanisms that lead to p53 autoimmunity are still unknown. The most widely accepted view is that mutant p53 acts as a nonself autoantigen. In a selected population of 30 patients with ovarian carcinoma, immunohistochemical analysis of tumor sections showed that 13 were p53 antigen positive. From these, 9 patients (69%) were also p53 antibody seropositive. Of the remaining 17 negative for p53 antigen tumors, 7 patients (41%) were serum p53 antibody positive. These data suggest that (1) patients are more frequently p53 antibody seropositive when their tumors exhibit p53 protein overexpression, and (2) tumors can elicit an antibody response even when they are p53 antigen negative as judged by immunohistochemistry. These findings have also been reported by others for ovarian carcinoma.[12] In a study by Green et al.,[12] 14 of 38 patients with ovarian carcinoma were p53 antigen positive. Of these patients, 7 (50%) were p53 antibody positive. Among 24 p53 antigen negative patients, 4 (25%) were p53 antibody positive. These data parallel the data reported in our study. At least three possibilities exist to explain the p53 autoantibody generation in patients whose tumors do not overexpress p53 protein: (1) the immune response is triggered by minute amounts of p53 protein, (2) focal p53 protein overexpression was missed during tumor sampling, and (3) tumors that were originally p53 antigen positive progressed to become p53 antigen negative.

In conclusion, we present data showing that a significant proportion of patients with ovarian carcinoma develop autoantibodies against p53 protein. Antibody generation was found more frequently in patients who had p53 protein overexpression in the tumor. However, p53 protein overexpression seemed not to be a necessary event for the autoimmune response. Presence of p53 autoantibodies was found to be associated with more aggressive tumors and decreased patient relapse free survival in univariate analysis. The independent prognostic value of the test, however, is limited.

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