

Mutant p53 Protein Overexpression Is Associated with Poor Outcome in Patients with Well or Moderately Differentiated Ovarian Carcinoma

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Background. It has been shown that the p53 gene is mutated in 30–80% of ovarian carcinomas and that the genetic alterations most often manifest as an accumulation of mutant p53 protein in tumor tissue. The prognostic significance of these findings for patients with ovarian cancer, however, must be established clearly.

Methods. Mutant p53 protein in 90 consecutive epithelial ovarian carcinomas was quantitatively analyzed using a time-resolved immunofluorometric procedure. In contrast to immunohistochemical techniques, this method uses two anti-p53 antibodies. The Cox model was used to evaluate the strength of the associations between the prognostic markers and disease relapse or death at univariate and multivariate levels. Kaplan–Meier survival curves were calculated for patients who were p53-positive or negative and for subgroups with a different clinical stage, histologic grade, or residual postsurgical tumor.

Results. The positivity rates for p53 included 1/21 (5%) with Stage I disease, 1/6 (17%) with Stage II, 29/51 (57%) with Stage III, and 8/12 (67%) with Stage IV (total = 39/90, 43%). Patients with p53-negative tumors had a significantly longer disease free survival than did patients with p53-positive tumors ($P = 0.03$); these results were similar for overall survival ($P = 0.06$). Multivariate analysis revealed that the presence of postsurgical residual tumor was the only predictor significantly associated with poor patient outcome. However, when patients were

divided into groups based on histologic grade, patients with well (G1) and moderately (G2) differentiated tumors had a significantly higher risk of cancer relapse and death if mutant p53 protein was present in their tumors compared with patients who were negative for mutant p53 protein (<0.01).

Conclusions. The immunofluorometric measurement of mutant p53 protein accumulation in epithelial ovarian carcinomas of a low histologic grade was associated significantly with an increased risk for cancer relapse and death. A similar trend also was suggested for early stage disease and in the absence of residual tumor after surgery. These increased risks, however, were not found for patients with high grade or advanced stage cancer or for those with residual tumor. To the authors' knowledge, this is the first report suggesting that p53 tumor protein accumulation is a marker of poor prognosis in a subset of patients with ovarian cancer. *Cancer* 1995;75:1327–38.

Key words: protein p53, ovarian neoplasms, enzyme-linked immunosorbent assay, survival, tumor antigens.

The p53 gene is classified as a tumor suppressor because it encodes a 393-amino acid nuclear phosphoprotein, which acts to restrain inappropriate cellular proliferation.^{1,2} The p53 protein is a transcription factor that binds to specific regions of DNA and regulates the expression of other genes.^{3,4} Loss of functional p53 protein due to point mutation and allelic deletion contributes to unrestrained cellular proliferation associated with cancer. Mutant p53 proteins have a prolonged half-life, accumulate in the nucleus, and can be detected by immunohistochemistry or other immunologic techniques.^{5,6} Mutation of the p53 gene is the most common genetic alteration yet revealed in human cancers.^{5,7}

Mutations in the p53 gene have been identified in a wide range of malignancies, including those of the colon, lung, breast, esophagus, endometrium, pros-

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tate, the hematopoietic system, and head and neck, as well as soft tissue sarcomas.^{1,5,8-12} This suggests that alteration of the p53 gene is a critical step in human carcinogenesis and that restoration of normal p53 gene function might become an integral component of generalized cancer treatment. These considerations explain why, over the last 3 years, the study of the p53 gene has become one of the most active fields in cancer research.

Epithelial ovarian cancer is the most lethal gynecologic malignancy.¹³ The high mortality rate associated with this tumor is due in large part to the absence of symptoms in the majority of women with early stages of the disease. Seventy percent of women present with advanced disease in which the tumor has spread to the peritoneal surface of the upper abdomen. Extensive intraabdominal disease is difficult to eradicate surgically, and many patients have only a partial response to postoperative chemotherapy. Although the molecular mechanisms of ovarian carcinogenesis still remain unknown, deletions of the region of chromosome 17p that encompasses the p53 gene are frequent.^{14,15} Previous studies have shown that the p53 gene is mutated in 30–80% of ovarian carcinomas and that the genetic changes correlate with mutant p53 protein accumulation in tumor tissue.¹⁶⁻²⁶

Although a consistent picture now is emerging to indicate that the loss of p53 function is associated with shortened survival in patients with breast, lung, gastric, endometrial, prostate, and cervical cancer,^{10,11,27-30} the data on the prognostic relevance of p53 gene mutation and protein accumulation in ovarian cancer are less abundant and also inconclusive.¹⁶⁻²⁶ Only one study in the literature was able to clarify partially the relationship among p53 expression, clinical outcome, and clinicopathologic features in human epithelial cancer of the ovary.³¹ Recently, the accumulation of p53 protein clearly has been shown to be an independent marker of reduced survival, flagging breast cancer cases for which more aggressive adjuvant therapy may be warranted.^{11,30,32} In the present study, the accumulation of p53 protein was considered an indicator of p53 gene mutation. Using a quantitative, highly sensitive time-resolved immunofluorometric procedure previously described by us,⁶ we evaluated the p53 protein accumulation in relation to patient survival and other prognosis-related clinicopathologic variables in 90 consecutive cases of epithelial ovarian carcinoma.

Patients and Methods

Study Population

Ninety-five consecutive patients with primary epithelial ovarian cancer were included in this study. All pa-

tients were treated and followed-up at the Department of Gynecologic Oncology of the University of Turin (Turin, Italy) between 1989 and 1993. Five patients (one with lymphoma of the ovary, two with primary breast cancer, one with primary colon cancer metastatic to the ovary, and one who was lost to follow-up) were excluded from this study. The age range of these patients was 20–78 years, with a median of 54 years. Follow-up time ranged from 1.3 months to 55.2 months, with a median of 22.2 months. All patients were staged according to the criteria of the International Federation of Gynecologists and Obstetricians.³³ The Federation's staging system assumes that an adequate staging operation has been performed.¹³ Staging operations included collection of ascites or peritoneal washings from the pelvis, gutters, and diaphragms for cytologic studies; total abdominal hysterectomy plus bilateral salpingo-oophorectomy; infracolic omentectomy and appendectomy; selective pelvic and paraaortic lymphadenectomy; and debulking of all gross disease. If obvious macroscopic tumor was not present, the following procedures were performed: biopsy of any lesion suspected of being a tumor metastasis or any adhesion adjacent to the primary tumor; blind biopsies of bladder peritoneum and cul-de-sac, right and left paracolic gutter, and pelvic side walls; and biopsy or smear of right hemidiaphragm.

All patients were treated with cisplatin-containing chemotherapeutic regimens unless otherwise indicated by the protocol used. Patients with Stage IA/IB and Grade 1 disease did not receive any further treatment. Twenty-one patients had Stage I disease, 6 patients had Stage II disease, 51 patients had Stage III disease, and 12 patients had Stage IV disease. Each tumor also had been histologically typed and graded based on World Health Organization³⁴ criteria and reviewed by a single pathologist. Sixteen tumors were Grade 1, 25 were Grade 2, and 49 were Grade 3. With respect to histologic type, 7 tumors were clear cell, 21 were endometrioid, 8 were mucinous, 36 were serous, 10 were undifferentiated, and 8 were unclassified. In the data analysis, according to histologic type, only endometrioid and serous cell carcinomas were considered. Because the number of samples of the other histologic types were too small to be analyzed reliably, they were combined together as one category called "others."

Tumor Extraction

Tumor specimens obtained during surgery were snap-frozen in liquid nitrogen and stored at –80°C until the time of analysis. About 200 mg of tumor tissue, which contained more than 70% tumor cells as determined by histologic examination, was pulverized to a fine powder

at -80°C , and the cells were lysed for 30 minutes on ice with 2 ml of lysis buffer (50 mmol/l Tris buffer [pH 8.0] containing 150 mmol sodium chloride, 5 mmol ethylenediaminetetra-acetic acid (EDTA), 10 g Nonidet NP-40 surfactant, and 1 mmol phenylmethylsulfonyl fluoride per liter). The lysate was centrifuged at 15,000 g at 4°C for 30 minutes, after which the supernatant was collected and immediately assayed for p53 protein, as well as for total protein with a commercial kit (Pierce Chemical Co., Rockford, IL) based on the bicinchoninic acid method.

Immunofluorometric Assay of Mutant p53 Protein

Mutant p53 in the tumor extracts was analyzed quantitatively with a time-resolved immunofluorometric procedure previously described elsewhere.⁶ Briefly, the tumor extracts were incubated with a mouse monoclonal anti-p53 antibody (PAB240, mutant-specific) in goat anti-mouse-coated polystyrene microtitre wells. After the wells were washed, a rabbit polyclonal anti-p53 antibody (CM-1, mutant- and wild-type specific) was added. After incubation and washing of the wells again, a goat anti-rabbit antibody conjugated to alkaline phosphatase was added. The activity of alkaline phosphatase was detected by the substrate diflunisal phosphate, which, when hydrolyzed and combined with a Tb^{3+} -EDTA solution, forms a fluorescent complex that can be measured with a time-resolved fluorometer.³⁵⁻³⁷ For quantitation, we used an arbitrary p53 standard solution established in our laboratory because no p53 standard is currently available. All p53 concentrations in the extracts, in arbitrary units per liter, were transformed to units of p53 per gram of total protein (U/g) to compensate for the amount of cells extracted per tumor.

Assignment of p53-Positivity Status

Examination of the distribution of p53 levels in all ovarian tumor extracts revealed two distinct populations with either relatively very low p53 levels (≤ 2 U/g; $n = 44$ samples) or clearly increased p53 levels (≥ 5 U/g; $n = 36$ samples). Only 10 samples were in the region between 2–5 U/g. For statistical analysis, we chose a cutoff level of 3 U/g, which separates the tumors into two groups: p53 negative ($n = 51$ [57%]) and p53 positive ($n = 39$ [43%]). This positivity rate is in close agreement with previously published positivities in which immunohistochemical or genetic techniques were used.^{15,17-20,22,26}

Statistical Analysis

For survival analysis, two different end points of follow-up—cancer relapse (either local recurrence or dis-

tant metastasis) and death—were used to calculate disease free and overall survival, respectively. Disease free survival was defined as the time interval between the date of surgery and the date of identification of recurrent or metastatic disease. Overall survival was defined as the time interval between the date of surgery and the date of death. The associations between p53 and other prognostic markers and between the prognostic markers and disease relapse or death were examined initially with the chi-square test.

The Cox proportional hazard regression model³⁸ was used to evaluate the strength of the associations (i.e., the hazard ratio and its confidence interval) between the prognostic markers and disease relapse or death. This analysis was conducted at both univariate and multivariate levels. In the multivariate analysis, the presence of mutant p53 protein, clinical stage (which was not included in overall survival analysis because the model did not converge), histologic grade, residual tumor, and age were included in the model. All of these variables except age were categorized dichotomously (i.e., p53-positive vs. p53-negative, clinical Stage I/II vs. Stage III/IV, histologic grade G1/G2 vs. grade G3, and the presence of residual tumor vs. the absence of residual tumor).

Kaplan–Meier survival curves³⁹ were constructed for p53-positive and p53-negative patients. Within each p53 category, Kaplan–Meier curves also were calculated for subgroups with different clinical stage, histologic grade, or residual tumor. The log rank test was used to examine the differences between the Kaplan–Meier curves.⁴⁰ Computer software SAS (SAS Institute, Cary, NC) and EGRET (Statistics and Epidemiology Research Corp., Seattle, WA) were used for these data analyses. Differences were considered significant when the probability values obtained from the statistical tests were 0.05 or less.

Results

The frequency distribution of p53 concentrations in the 90 patients studied is shown in Figure 1. Of these 90 patients, 43% overexpressed p53 protein. One tumor that was highly positive for p53 protein by the immunologic assay and one tumor that was p53 negative were stained immunohistochemically with the monoclonal anti-p53 antibody DO-1, as described by Vojtesek et al.⁴¹ These data, presented in Figure 2, confirm the concordance between our immunologic assay and immunohistochemistry, demonstrated previously by other investigators.⁴¹⁻⁴³ We also extracted DNA from six tumors, amplified the p53 gene, and performed single-strand conformation polymorphism analysis of exons 5–8, as described by Mashiyama et al.⁴⁴ DNA from the

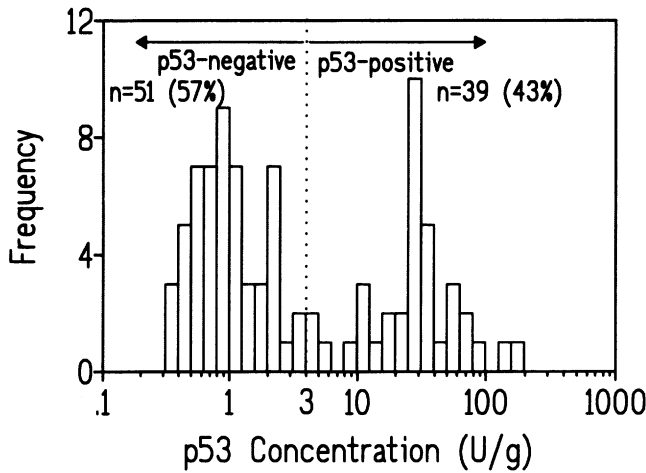


Figure 1. Frequency distribution of p53 concentration in 90 ovarian tumor extracts. The dotted line indicates the cutoff point used.

colon carcinoma cell line COLO320 HSR (+), which overproduces mutant p53 and has a point mutation in exon 7 of the p53 gene also was included as a positive

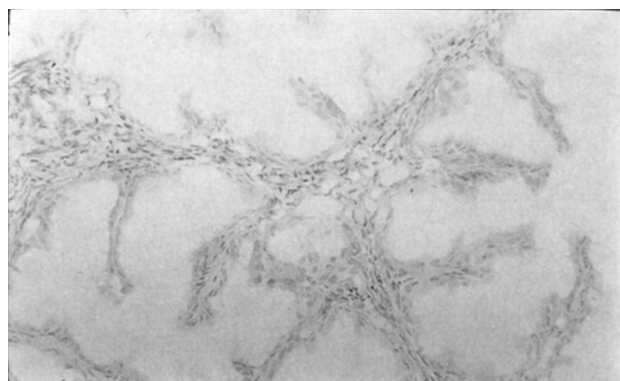
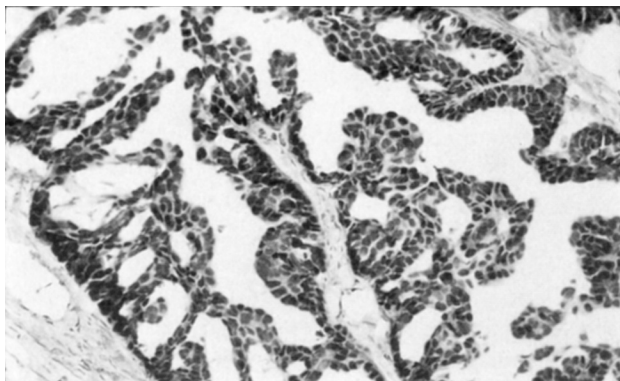


Figure 2. Immunohistochemical staining of an ovarian tumor determined positive for p53 by the immunologic assay (top) and a tumor negative for p53 (bottom). Staining was performed with the DO-1 monoclonal anti-p53 antibody and avidin-biotin-peroxidase techniques.⁴¹

Table 1. Associations Between p53 and Clinicopathologic Variables

Clinicopathologic variable	p53 negative	p53 positive	P value
Age (yr)			
<40	8 (15.7)	2 (5.1)	
40-49	14 (27.5)	7 (18.0)	
50-59	16 (31.4)	17 (43.6)	
60+	13 (25.4)	13 (33.3)	0.22
Menopause*			
Pre	25 (49.0)	11 (29.0)	
Post	26 (51.0)	27 (71.0)	0.06
Stage			
I-II	25 (49.0)	2 (5.1)	
III-IV	26 (51.0)	37 (94.9)	<0.01
Grade			
1	14 (27.5)	2 (5.1)	
2	15 (29.4)	10 (25.7)	
3	22 (43.1)	27 (69.2)	0.01
Residual tumor (cm)†			
0	28 (54.9)	6 (15.8)	
≤5	14 (27.5)	16 (42.1)	
>5	9 (17.6)	16 (42.1)	<0.01
Histotype			
Endometrioid	19 (37.3)	2 (5.1)	
Serous	14 (27.5)	22 (56.4)	
Others	18 (35.2)	15 (38.5)	<0.01

Values are no. of patients (%).

* Menopausal status unknown for one patient.

† Residual tumor unknown for one patient.

control. The colon carcinoma cell line and four ovarian tumors were found to possess p53 gene mutations in exons 7, 5-6, 8, 8 and 7, respectively, and also to overproduce p53 protein as measured by the immunologic assay. Two tumors in which we did not detect any p53 gene mutations in exons 5-8 had levels of p53 protein below the cutoff level of 3 U/g.

Table 1 presents the relationships between p53 and other clinical or pathologic variables, including age, menopausal status, clinical stage, histologic grade, residual tumor after surgery, and histologic type. Patients with p53-positive tumors tended to be older than patients with p53-negative tumors, but the difference was not statistically significant. Of all p53-positive patients, 77% were 50 years of age or older. In the p53-negative group, only 57% of patients were in the same age category. Similar tendency also was observed between p53 and menopausal status, because menopause is an age-dependent event. The presence of mutant p53 protein was associated significantly with late clinical stage, high histologic grade, presence of residual tumor, and serous histologic type (Table 1).

The positivity rates for p53 per stage were 5% (1 of 21) for Stage I disease, 17% (1 of 6) for Stage II, 57%

Table 2. Associations Between Clinicopathologic Variables, p53, and Cancer Relapse or Death*

Variable	Relapse	Relapse-free	P value	Dead	Alive	P value
Age (yr)						
<40	2 (5.7)	8 (14.6)		2 (7.4)	8 (12.7)	
40–49	8 (22.9)	13 (23.6)		7 (25.9)	14 (22.2)	
50–59	13 (37.1)	20 (36.4)		7 (25.9)	26 (41.3)	
60+	12 (34.3)	14 (25.4)	0.55	11 (40.7)	15 (23.8)	0.30
Menopause†						
Pre	10 (28.6)	26 (48.2)		9 (33.3)	27 (43.6)	
Post	25 (71.4)	28 (51.8)	0.07	18 (66.7)	35 (56.4)	0.37
Stage						
I–II	3 (8.6)	24 (43.6)		0	27 (42.9)	
III–IV	32 (91.4)	31 (56.4)	<0.01	27 (100)	36 (57.1)	<0.01
Grade						
1	1 (2.9)	15 (27.3)		2 (7.4)	14 (22.2)	
2	12 (34.3)	13 (23.6)		6 (22.2)	19 (30.2)	
3	22 (62.8)	27 (49.1)	0.01	19 (70.4)	30 (47.6)	0.10
Residual tumor (cm)‡						
0	4 (11.8)	30 (54.6)		1 (3.9)	33 (52.4)	
≤5	15 (44.1)	15 (27.3)		10 (38.5)	20 (31.8)	
>5	15 (44.1)	10 (18.1)	<0.01	15 (57.6)	10 (15.8)	<0.01
Histotype						
Endometrioid	3 (8.6)	18 (32.7)		2 (7.4)	19 (30.2)	
Serous	16 (45.7)	20 (36.4)		11 (40.7)	25 (39.6)	
Others	16 (45.7)	17 (30.9)	0.03	14 (51.9)	19 (30.2)	0.04
p53						
Positive	20 (57.1)	19 (34.6)		16 (59.3)	23 (36.5)	
Negative	15 (42.9)	36 (65.4)	0.04	11 (40.7)	40 (63.5)	0.05

Values are no. of patients (%).

* In this analysis, the follow-up time for each patient was not taken into consideration.

† Menopausal status unknown for one patient.

‡ Residual tumor unknown for one patient.

(29 of 51) for Stage III, and 67% (8 of 12) for Stage IV. Similarly, the positivity rates for p53 per grade were as follows: 13% (2 of 16) for G1, 40% (10 of 25) for G2, and 55% (27 of 49) for G3. The positivity rates for p53 were 18% (6 of 34) in patients with no residual tumor after surgery, 53% (16 of 30) in patients with a residual tumor 5 cm or smaller in size, and 64% (16 of 25) in patients with residual tumor larger than 5 cm. The positivity rates for p53 were 29% (2 of 7) for clear cell tumors, 10% (2 of 21) for endometrioid tumors, 38% (3 of 8) for mucinous tumors, 61% (22 of 36) for serous tumors, 50% (4 of 8) for unclassified tumors, and 60% (6 of 10) for undifferentiated tumors.

The associations between these prognostic markers and cancer relapse or death are shown in Table 2. Statistically significant associations were not seen between cancer relapse or death and patient age or menopausal status, although there was a trend for older or postmenopausal patients to suffer a relapse or die more frequently. Clinical stage, histologic grade, histologic type, and postsurgical residual tumor all were associated significantly with cancer relapse or death. Patients whose tumors were p53 positive also had higher relapse and

death rates in comparison with patients whose tumors were p53 negative, and the differences were statistically significant (Table 2). Patients with p53-negative tumors had significantly longer disease free survival compared with patients with p53-positive tumors (Fig. 3). A similar tendency also was seen for overall survival, with a border-line statistical significance ($P = 0.06$).

The strength of the associations between each individual predictor and disease free or overall survival are demonstrated in the univariate analysis in Table 3. The presence of residual tumor showed the strongest association with cancer relapse and death, and the hazard ratio reached maximum values of 8.3 and 27.5, respectively. Patients with late clinical stage (III or IV) or poorly differentiated (G3) tumors had a two-to-seven times higher risk of developing recurrent or metastatic disease or of dying compared with those with early stage (I or II) or well or moderately differentiated (G1 or G2) tumors. The hazard ratio for patients with p53-positive tumors was 2 for both disease free and overall survival.

When all these predictors were included in the Cox model (multivariate analysis in Table 3), however, the

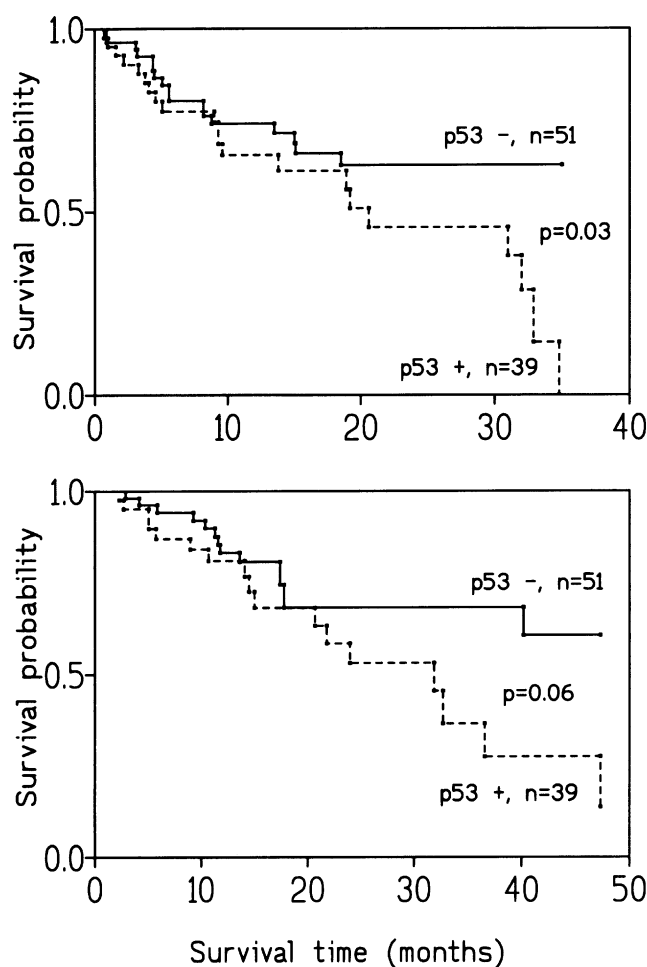


Figure 3. Kaplan-Meier survival analysis of disease free (top) and overall survival (bottom) in patients with ovarian cancer who were either p53 negative (p53-) or p53 positive (p53+). n: number of patients.

presence of residual tumor was the only predictor significantly associated with disease free and overall survival. Mutant p53 protein, clinical stage, and histologic grade were shown to have no independent value for predicting the prognosis of ovarian cancer patients.

We further examined the associations between mutant p53 protein and cancer relapse or death in subgroups of patients who were categorized based on either their clinical stage (I/II vs. III/IV), histologic grade (G1/G2 vs. G3), or postsurgical residual tumor (presence vs. absence). The results are shown in Table 4. Patients with well or moderately differentiated cancer had a significantly higher risk of developing recurrent disease or dying if mutant p53 protein was present in their tumor when compared with patients who had tumors of the same grade but were p53-negative. Patients with no postsurgical residual tumor also demonstrated increased risk for relapse and death when the tumors

were p53 positive. Although higher risks for relapse and death also were observed in patients with p53-positive tumors who had disease of an early clinical stage, the elevated risk did not reach statistical significance. The risk for cancer relapse or death was not significantly different between p53-positive and p53-negative tumors in patients with late clinical stage, poorly differentiated tumors, or postsurgical residual tumor. These findings also were shown in Kaplan-Meier survival curves.

Figure 4 shows the disease free and overall survival curves for cancer patients with histologic grades G1 and G2. Patients with p53-negative tumors had substantially longer disease free and overall survival than did patients with p53-positive tumors ($P < 0.01$). These differences were not seen in patients with poorly differentiated cancer (Fig. 5). For patients with residual tumor after surgery (Fig. 6) or late clinical stage (Fig. 7), there was no difference in the disease free or overall survival curves between p53-positive and p53-negative tumors. Kaplan-Meier survival curves were not calculated for patients with early clinical stage cancer or with no residual tumor because of the small number of patients in each p53-positive group.

Discussion

More than 100 cancer-related genes have been discovered, several of which have been implicated in the natural history of human cancer because they consistently are found to be mutated in tumors. The p53 tumor suppressor gene is the most striking example because it is mutated in approximately half of almost all types of cancer arising from a wide spectrum of tissues.⁵ A number of clinical studies have examined the frequency of p53 mutations in epithelial ovarian carcinoma using immunohistochemical or genetic techniques.¹⁶⁻²⁶ With the exception of three reports,^{22,25,31} these studies suffer from two important limitations: (1) a small number of patients and (2) study of patients primarily with late stage disease. Therefore, no conclusive evidence could be presented on the prognostic significance of p53 in epithelial ovarian carcinoma. To the authors' knowledge, the present report is the first to indicate that p53 tumor protein accumulation is a marker of poor prognosis in a subset of ovarian cancer patients.

Previous studies have demonstrated that the positivity rate of p53 gene mutations or of p53 protein accumulation in ovarian cancers is between 15%²⁵ and 79%,²¹ but in the majority of studies, the positivity was approximately 50%.^{15,17,19,20,22,26} The apparent discrepancies can be explained by differences in the methodologies employed and the patients selected. It is now well accepted that there generally is a concordance between

Table 3. Cox Proportional Hazard Regression Analysis

Variable	Relapse			Death		
	HR	95% CI	P value	HR	95% CI	P value
Univariate analysis						
p53*	2.03	1.06–3.89	0.03	1.95	0.96–3.97	0.06
Age	1.02	0.99–1.05	0.10	1.03	1.00–1.07	0.04
Grade†	2.23	1.11–4.47	0.02	3.22	1.42–7.30	0.01
Stage‡	7.28	2.20–24.11	<0.01	0		
Residual tumor§	8.31	2.90–23.83	<0.01	27.48¶	2.70–204.4	<0.01
Multivariate analysis						
p53*	0.84	0.41–1.72	0.63	0.86	0.40–1.87	0.72
Age	1.03	0.99–1.06	0.11	1.04	1.00–1.09	0.05
Grade†	0.79	0.38–1.67	0.54	1.00	0.42–2.34	0.99
Stage‡	2.69	0.55–13.15	0.22	0		
Residual tumor§	5.35	1.42–20.14	0.01	30.89¶	3.83–248.8	<0.01

HR: hazard ratio; CI: confidence interval.

* p53 presence versus p53 absence.

† Grade 1 and Grade 2 versus Grade 3.

‡ Stage I and II versus Stage III and IV.

§ Residual tumor presence versus residual tumor absence.

|| No death in the group of patients with Stage I–II.

¶ Only one death in the group of patients with no residual tumor.

genetic methods that detect p53 gene mutations and immunologic methods that measure mutant p53 protein accumulation.^{21,25} Immunohistochemical methods, however, do not detect genetic changes that are either nonsense or frameshift mutations, because the truncated mutant proteins usually are not reactive with the anti-p53 antibodies used. Conversely, genetic methods frequently screen only exons 5–8 for p53 gene mutations, but mutations in other exons also may occur, al-

though with low frequency.²¹ Additionally, there is also a possibility of that p53 protein is stabilized through nonmutational mechanisms or p53 protein overexpression because of mutations in the promoter region.^{45,46} Because immunohistochemical techniques are interpreted by somewhat subjective criteria and different antibodies against p53 are often used in these procedures, the results from different investigators may vary.

Table 4. Cox Proportional Hazard Regression Analysis for Subgroups of Patients

Variable	Relapse			Death		
	HR	95% CI	P value	HR	95% CI	P value
Grade I–II						
p53 unadjusted	11.07	3.28–37.34	<0.01	25.03	3.06–204.7	<0.01
p53 age-adjusted	9.43	2.73–32.54	<0.01	20.67	2.52–169.3	0.01
Grade III						
p53 unadjusted	0.54	0.24–1.24	0.15	0.48	0.20–1.17	0.11
p53 age-adjusted	0.55	0.24–1.26	0.16	0.46	0.18–1.13	0.09
Stage I–II						
p53 unadjusted	5.82	0.51–66.81	0.16	0*		
p53 age-adjusted	8.43	0.46–154.2	0.15	0*		
Stage III–IV						
p53 unadjusted	1.04	0.53–2.05	0.90	0.97	0.47–1.98	0.92
p53 age-adjusted	0.94	0.46–1.90	0.86	0.76	0.35–1.62	0.76
No residual tumor						
p53 unadjusted	14.17	1.45–138.3	0.02	0†		
p53 age-adjusted	8.49	0.85–85.08	0.07	0†		
Residual tumor						
p53 unadjusted	0.71	0.36–1.43	0.34	0.91	0.42–1.94	0.80
p53 age-adjusted	0.69	0.34–1.37	0.29	1.04	0.38–1.76	0.60

HR: hazard ratio; CI: confidence interval.

* No death in the group of patients with Stage I–II.

† Only one death exists in the group of patients with no residual tumor, and convergency could not be achieved in the calculation of model parameters.

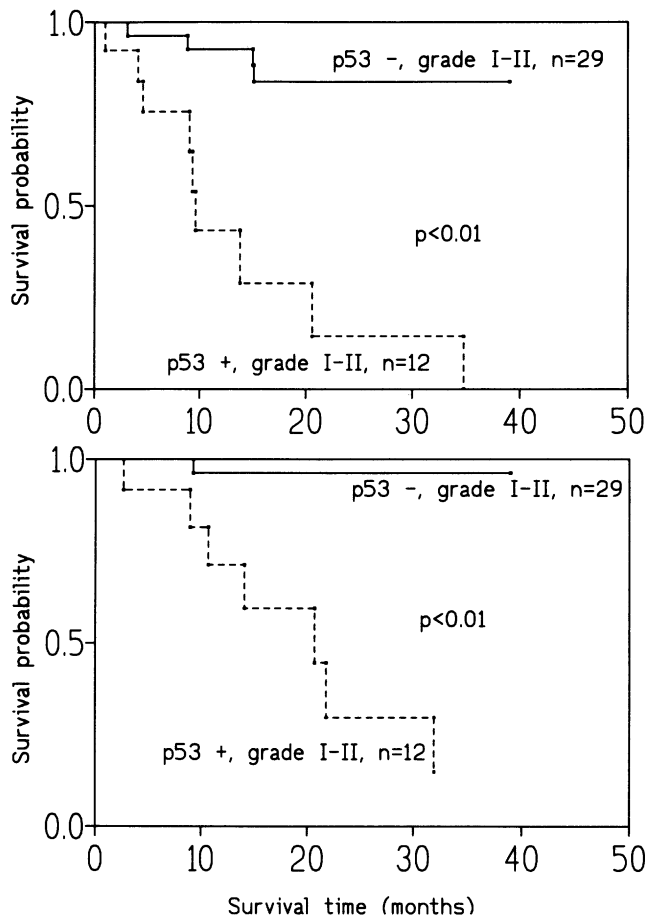


Figure 4. Kaplan-Meier survival analysis of disease free (top) and overall survival (bottom) in patients with Grade I/II ovarian cancer who were either p53 negative (p53-) or p53 positive (p53+). n: number of patients.

Our immunologic assay for p53 protein quantification has been reported recently.⁶ Other groups have developed similar assays,⁴¹⁻⁴³ and one method is commercially available (Oncogene Science Inc., Uniondale, NY). During the evaluation of this assay, we have shown that cell lines bearing p53 gene mutations overexpress p53 protein, whereas cell lines with normal p53 gene do not. Using this assay we also were able to confirm the negative associations between p53 gene mutations and steroid hormone receptors in breast cancer.⁶ The advantages of quantitative immunologic assays over immunohistochemical techniques recently have been summarized.⁴⁷ Immunologic assays for p53 mutant protein quantification have been compared with immunohistochemical and genetic techniques. The data were highly correlated in all studies.^{6,41,43} Our limited immunohistochemical and genetic data with single-strand conformation polymorphism analysis confirm these correlations.

In regard to patient selection, most published stud-

ies that examined p53 gene mutations in ovarian cancer included patients mostly with Stages III or IV disease.²⁰⁻²³ It has been shown that p53 gene mutations in ovarian cancer appear with lower frequency in early stages of the disease.^{16,23} An extensive study by Marks et al.²² initially failed to show differences in the p53 gene mutation rate at various disease stages. In a subsequent study,²⁵ however, the same group found that the mutation rate in Stage IA/IB disease was 15% and that the rate increased to 44% and 56% in Stages IC/II and III disease, respectively. In our study, we demonstrated a similar trend in the positivity rate for mutant p53 protein (i.e. 5%, 17%, 57%, and 67% in Stages I, II, III, and IV, respectively). Other factors that may affect the p53 positivity rate in ovarian cancer include histologic grade, histologic type, and the presence of postsurgical residual tumor.

The increase in p53 positivity rate with disease stage may indicate either that aberrant p53 expression may be a relatively late event in ovarian carcinogenesis

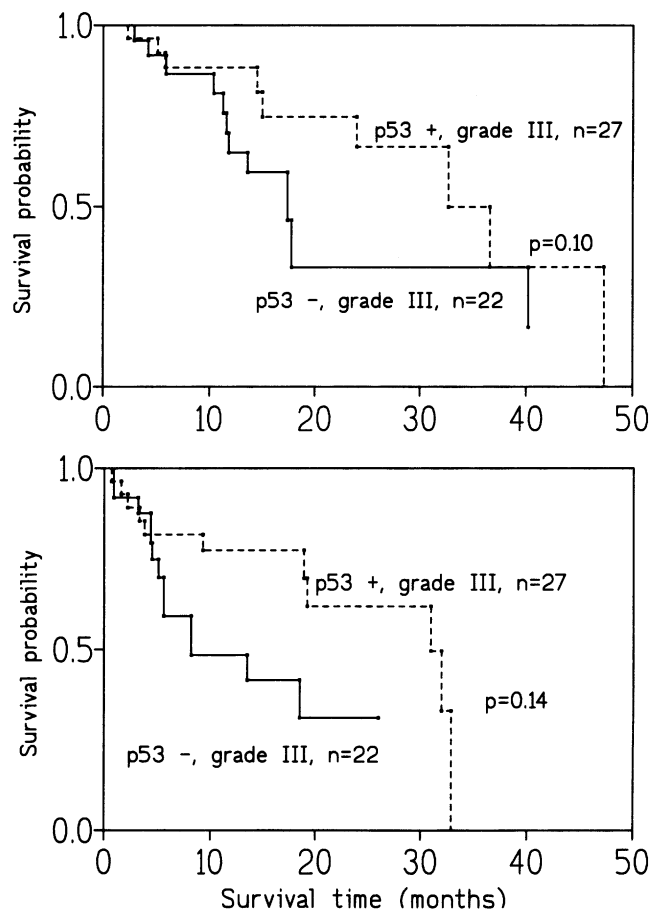


Figure 5. Kaplan-Meier survival analysis of disease free (top) and overall survival (bottom) in patients with Grade III ovarian cancer who were either p53 negative (p53-) or p53 positive (p53+). n: number of patients.

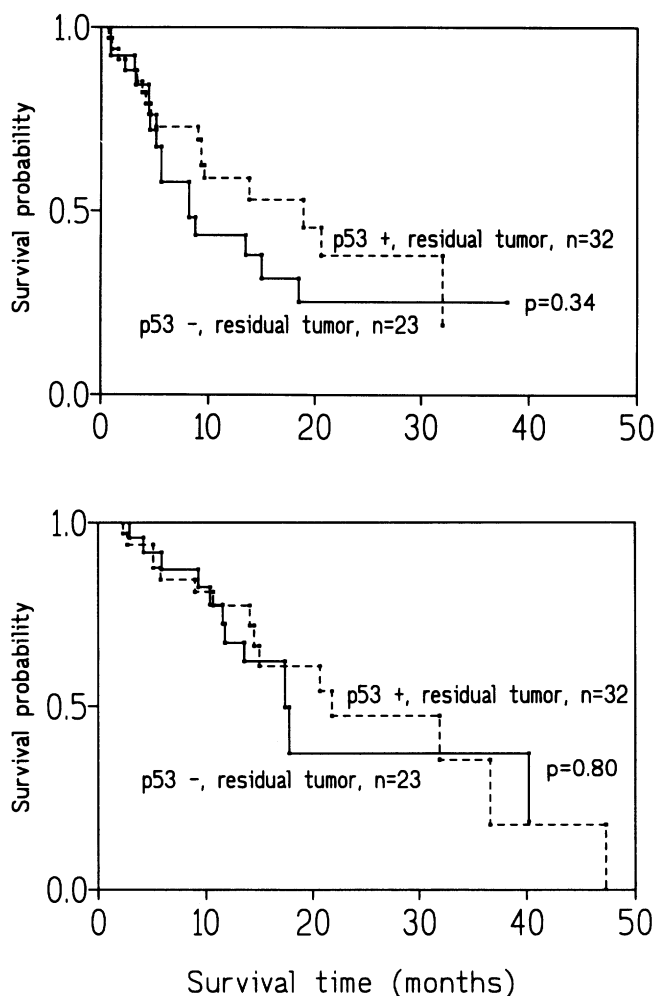


Figure 6. Kaplan-Meier survival analysis of disease free (top) and overall survival (bottom) in patients with residual tumor after cytoreductive surgery who were either p53 negative (p53-) or p53 positive (p53+). n: number of patients.

that occurs during tumor progression and metastasis, or that ovarian cancer without p53 gene mutations represents a different and perhaps less aggressive subset of ovarian cancers that have decreased ability to metastasize.

It is thought that mutations observed in ovarian cancer are due to errors in DNA synthesis that occur at a low frequency during DNA replication associated with normal cellular proliferation.⁴⁸ Therefore, the longer one is alive, the higher the probability of accumulating cells that have acquired the multiple activated cancer-causing genes required for the development of a clinically recognizable tumor. Data from other tumors appear to indicate that activation of p53 may occur at different stages of tumorigenesis (early or late event) in different tumor types.⁴⁹⁻⁵¹ The accumulation of multiple activated genes may be more important than the order in which they occur.

In this study, we used a cutoff level of 3 U/g to divide the patients into two groups, p53 positive and p53 negative. Based on the frequency distribution of the p53 values among all the patients (Fig. 1), we found that only 10% of the patients had a p53 value close to the cutoff level. The majority of the p53-positive patients had values much higher than 3 U/g.

The highest percentage of p53-positive tumors were associated with serous histologic type (61%), followed by undifferentiated (60%), unclassified (50%), mucinous (38%), clear cell (29%), and endometrioid (10%) tumors. This finding is in agreement with previous reports that also demonstrated that the highest p53 positivity was associated with serous tumors, whereas endometrioid tumors had a relatively low p53 positivity.^{20,21,23}

Initial univariate analysis revealed that the detec-

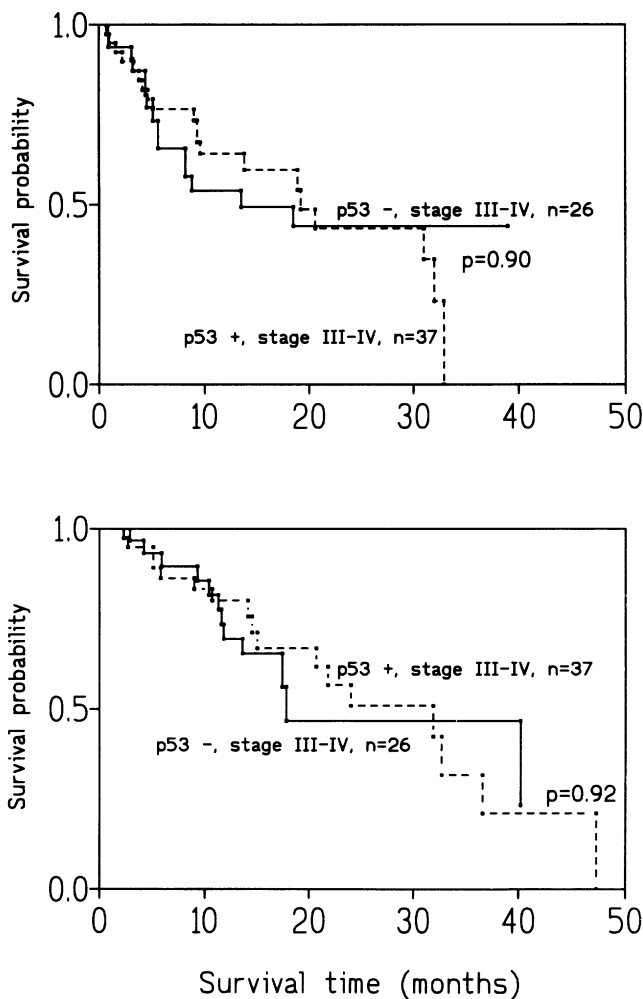


Figure 7. Kaplan-Meier survival analysis of disease free (top) and overall survival (bottom) in patients with Stage III/IV ovarian cancer who were either p53 negative (p53-) or p53 positive (p53+). n: number of patients.

tion of mutant p53 in ovarian cancer was significantly associated with increased frequency of relapse and death. Patients with late disease stage, poorly differentiated tumors, or residual cancer diagnosed after surgery also had significantly higher risk for relapse and death. In multivariate analysis, however, only the presence of postsurgical residual tumor was associated significantly with survival, whereas none of the other markers showed any independent predictive value for patient prognosis. Such predictive value may be established at least for some parameters if longer follow-up periods are evaluated.³¹

Because postsurgical residual tumor, clinical stage, and histologic grade all were associated significantly with the presence of p53 and survival outcome, we considered the possible existence of interactions (or effect modifications) of these predictors on patient survival, because such interactions may lead to improper observation of the associations of these markers with the survival. To avoid the interaction between the predictors on the survival and simultaneously to control for the confounding effect of other predictors when the predictive value of p53 was examined, we evaluated the relationship between mutant p53 protein and survival in patients who were subclassified into groups based on their clinical stage, histologic grade, and presence of postsurgical residual tumor. In this analysis, we found a strong and highly significant association between the presence of mutant p53 protein and increased cancer relapse or death rates in well or moderately differentiated tumors (G1 or G2), but not in poorly differentiated tumors (G3). This association also was observed in the subgroup of patients who had no postsurgical residual tumor, but not in the subgroup of patients who had residual tumor. Similar predictive value of p53 also seemed to be present in patients with early clinical stage disease (I or II) but not in those with late stage disease (III or IV). The above observations also were well demonstrated by the Kaplan–Meier survival curves.

With respect to the Kaplan–Meier survival curves plotted for the p53-positive and p53-negative tumors without subclassification, it is evident that the curves begin to diverge after 20 months of follow-up, which may suggest that the differences are contributed by tumors with better prognosis (Fig. 3). This observation provides support to the finding that patients with well or moderately differentiated cancer had better prognosis if mutant p53 protein was absent.

Kaplan–Meier survival plots could not be constructed for the subgroups of patients with Stage I–II disease or with no residual tumor postcytoreductive therapy because of the small number of patients in each p53-positive group who developed cancer relapse or died. The data shown in Table 4, however, indicate that

p53 also may be a predictor of poor outcome in early clinical stage tumors or in patients without residual tumor after surgery, confirming recently published results.³¹ These suggestions need to be confirmed with more patients or with longer follow-up periods.

To our knowledge, this study is the first to demonstrate a statistically significant association between the presence of mutant p53 protein and poor outcome in a subset of ovarian cancer patients who have either well or moderately differentiated ovarian carcinoma ($P < 0.01$). All but one³¹ previous study that examined the association of p53 gene mutations and clinical outcome enrolled a small number of patients with late stage disease. In the only study that examined p53 gene mutations in early ovarian cancer,²⁵ no association was found between p53 presence and overall survival, but the patients were not subclassified according to histologic grade. We suspect that the disagreement between our results and those previously published²⁵ may be due largely to patient selection and methodologic differences. This previously published study²⁵ reported the highest prevalence of p53-positive tumors in the mucinous histologic type (57%) and lower prevalence in the serous histologic type (30%), in contrast to our findings and those of others.^{20,21,23} Hartmann et al.³¹ found immunoreactivity for p53 in 62% of patients with early and advanced stage epithelial ovarian cancer and a statistically significant association between p53 staining, higher grade disease, and overall survival in univariate analysis. Our study confirms the trend found in this other study for a correlation of p53 positivity in early stage tumors with reduced patient survival.

Our findings prompt us to speculate that in tumors that are not well differentiated (G3), in addition to or independent of the presence of p53, other genes may confer an aggressive phenotype to the ovarian tumor that becomes lethal to the patient. These unknown genes apparently are not expressed in G1 or G2 tumors, and in this case, the unfavorable function of p53 becomes manifest. Alternatively, it is possible that ovarian neoplasms that are well or moderately differentiated and nonmetastatic at the time of diagnosis represent a different, less aggressive subset of ovarian tumors that is simply less likely to have acquired p53 gene mutations. The lack of an easily identifiable premalignant lesion in the ovarian epithelium, in comparison with colon and breast cancers, is a major obstacle in determining whether aberrant p53 expression is an early or late event in ovarian carcinogenesis. The molecular mechanisms by which mutant p53 contributes to the malignant phenotype remain under investigation.

It is recognized that most patients with ovarian cancer have advanced disease at the time of diagnosis and will receive postsurgical chemotherapy regardless of tu-

mor grade or other features. Our findings would be useful in guiding treatment decisions in the small number of patients who present with early stage/grade disease. In these patients, assessment of p53 gene alterations or p53 protein overexpression would allow physicians to treat those who are at higher risk for relapse and spare treatment for those with good prognosis.

In conclusion, we found that the presence of p53 in well or moderately differentiated ovarian cancer is a strong indicator of poor prognosis. In poorly differentiated or late stage ovarian carcinoma, however, other genes that remain to be identified may be responsible for the unfavorable prognosis.

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