Evidence for a Dose-Response Effect between p53 (but not p21$^{WAF1/Cip1}$) Protein Concentrations, Survival, and Responsiveness in Patients with Epithelial Ovarian Cancer Treated with Platinum-based Chemotherapy[1]

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ABSTRACT

The prognostic values of p53 and of its downstream mediator p21$^{WAF1/Cip1}$ in patients receiving adjuvant chemotherapy for epithelial ovarian cancer have not been clearly established. Tumor extracts from a series of 120 patients treated postsurgically with cisplatin or carboplatin alone or together with other chemotherapeutics for primary ovarian carcinoma were assayed both for p53 protein by an immunofluorometric assay developed by us and for p21 protein by a commercially available immunoassay. Relative risks (RRs) for cancer relapse and death after 24 months of follow-up were determined by multivariate Cox regression analysis. Disease-free (DFS) and overall survival (OS) probabilities were also examined by the Kaplan-Meier method and log-rank tests. All other procedures were similarly nonparametric and based on two-sided tests of significance. Concentrations of p53 were elevated in patients with advanced stage disease ($P = 0.02$) or poorly differentiated ($P = 0.03$), suboptimally debulked tumors ($P = 0.02$), as well as in patients who failed to respond to chemotherapy ($P = 0.03$), as assessed by computed tomography scanning, serum CA125 determination, and second-look laparotomy. Statistically significant associations between concentrations of p53 and p21 were not found, nor were relationships demonstrated between concentrations of p21 and other clinicopathological variables or treatment response. Univariate analysis showed that p53 concentrations above the median indicated significantly higher risks for relapse ($P = 0.04$) and death ($P < 0.01$) and showed trends for increasing risks for relapse ($P = 0.04$) and death ($P < 0.01$) when p53 was considered as a four-level categorical variable. Multivariate analyses adjusted for age, stage, grade, and residual tumor size confirmed these observations (RR = 1.50; $P = 0.05$ for DFS and RR = 1.92; $P = 0.03$ for OS) for median-dichotomized p53, but the trends were of borderline significance ($P = 0.09$ for DFS and $P = 0.07$ for OS). In contrast, p21 positivity was not a significant predictor of favorable outcome in univariate survival analysis, and use of a three-level variable combining positivity or negativity status for both p53 and p21 did not yield greater separation of patients into risk groups ($P = 0.07$ for DFS and $P = 0.06$ for OS) than the use of p53 alone. Assessment of p53 expression may be an independent indicator of poor prognosis in ovarian cancer patients treated with adjuvant chemotherapy. The prognostic value of p21 expression, however, could not be demonstrated in our series of ovarian cancer patients.

INTRODUCTION

Epithelial ovarian cancer is the most lethal gynecological malignancy in Western countries (1). Approximately 80% of patients are diagnosed with advanced stage disease (2), associated with a 5-year survival rate of only 30% despite improvements in long-term survival gained by the use of combination chemotherapy, principally with cisplatin and more recently with paclitaxel (3). A number of factors contribute to the poor prognosis of patients with advanced stage ovarian carcinoma, including the failure of aggressive cytoreductive surgery to completely eradicate metastatic disease in >75% of cases, the intrinsic resistance to adjuvant chemotherapy in over half of these patients, as well as the development of chemoresistance in nearly half of the initially responsive patients during the course of their treatment (4). Although clinicopathological characteristics of ovarian cancer other than disease stage, such as volume of residual disease after debulking surgery, histological grade and type, lymph node status, and presence of ascites are also of demonstrated prognostic value (5), individual patients may show significant differences in chemosensitivity although they share identical clinicopathological features. In light of evidence indicating that most anticancer agents induce tumor regression by triggering apoptosis (6), it is possible that new variables

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reflecting the apoptotic potential of ovarian neoplasms may offer more accurate prognostic information for patients treated with chemotherapy.

Among the determinants for the induction of some forms of apoptosis is the status of the *p53* tumor suppressor gene, the translated product of which has been shown to transcriptionally up-regulate and down-regulate bax (7) and bcl-2 (8), respectively, two key components of the triggering mechanism for programmed cell death. Functional loss of p53 by mutations that interfere with its ability to induce apoptosis has been shown to facilitate the development of neoplastic clones resistant to different chemotherapeutic drugs (9). These mutations, which are mostly missense and occur within conserved sequences of the *p53* gene, are the most common genetic alterations in human malignancy (10) and have been detected in ~50% of epithelial ovarian cancers (11). Rather than impeding p53 protein expression, missense mutations usually confer an altered conformation to the mutant p53 protein and are associated with its predominantly nuclear accumulation (12), in contrast to normal cell nuclei in which p53 protein is expressed at very low levels. Besides its diminished capacity to trigger apoptosis, mutant p53 protein is typically also deficient in its ability to induce cell cycle arrest by the transactivation of other target genes. The first identified of these was $p21^{WAF1/Cip1}$, a protein that binds and inhibits several cyclin/cyclin-dependent kinase complexes (13, 14) as well as components of the DNA replication machinery (15). Despite observations that expression of p21, like p53, can cause growth suppression of a variety of cell types *in vitro* (13, 16) and *in vivo* (17), p21 mutations rarely occur in human cancers (18, 19), suggesting that derangement of p21 function does not contribute to clinical disease. However, p21 protein expression has been shown to be highly variable in several tumor types (20–22) and to be subject to both p53-dependent and p53-independent transcriptional regulation (23). Unlike the large number of studies investigating the relationship between p53 alteration in diverse malignancies, including ovarian cancer (24–28), and unfavorable prognostic outcome, there have been fewer studies examining p21 expression in relation to patient prognosis (21, 22, 29–31). Moreover, to our knowledge, the prognostic and predictive implications of p53, considered together with its downstream mediator p21, in epithelial ovarian cancer have not yet been reported.

Conventional tools to identify p53 abnormalities have consisted of DNA sequencing methods, indirect screening methods for determining DNA sequence changes, and immunohistochemical staining techniques using monoclonal or polyclonal antibodies to detect p53 protein overexpression. The latter approach, although lacking sensitivity and specificity for demonstrating p53 changes at the genetic level (32), nonetheless has been shown to provide useful information regarding the prognosis of patients with ovarian carcinoma (25, 27, 28) at a fraction of the technical costs. Use of the same antibodies as reagents in immunoassays of p53 constitutes an alternative to p53 immunostaining that may offer advantages in terms of more objective results interpretation and relative ease of quantitation. Such immunoassays have been applied to the measurement of p53 concentrations in extracts prepared from a variety of tissues and have yielded results highly concordant with those obtained by immunostaining (33–35). Only immunohistochemical methods, however, have been used for the detection of p21 protein in clinical specimens (20–22, 29–31), despite the possible advantages of commercially available immunoassays.

In this study, we report the use of simple yet sensitive immunoassays of p53 and p21 proteins, rather than immunostaining, to determine their respective concentrations in extracts of 120 epithelial ovarian carcinomas obtained from chemotherapy-treated patients residing in the Piedmont region of Northern Italy. The expression levels of p53 and p21 were related to each other, to other prognostic features, to patient response to administered chemotherapy, and to DFS[3] and OS.

PATIENTS AND METHODS

Ovarian Cancer Patients. This study had been approved by the Ethics and Research Committees at the University of Toronto and the University of Turin that assured patient confidentiality at every stage of the investigation. One hundred and twenty consecutive patients with primary epithelial ovarian carcinoma, operated at the Department of Gynecology, Gynecological Oncology Service of the University of Turin, Turin, Italy between April 1988 and January 1997, were included in this study. Excluded from this consecutive series had been patients with benign ($n = 4$) or germ-line ($n = 4$) ovarian neoplasms, patients with other primary malignancies metastatic to the ovary ($n = 19$), and patients with cancer of the ovarian epithelium who had tumor specimens that were either of borderline histological grade ($n = 16$) or of insufficient quantity for p53 protein analysis ($n = 6$). Three patients were lost to follow-up, and four who did not receive adjuvant chemotherapy were also excluded. The patients studied were of ages ranging from 26 to 77 years; the median and mean ages were both 55 years. Patients were followed up at the same institution for periods ranging from 3 to 119 months (median and mean were 24 and 30 months, respectively), during which 66 (55%) were diagnosed with ovarian cancer relapse and 44 (37%) died of their disease. DFS time, defined as the number of complete months elapsed from the date of tumor resection to that of the first evidence of recurrent disease or distant metastasis in each case, was distributed from 1 to 67 months with a mean and median of 15 and 11 months, respectively. Of the 66 patients who relapsed, 12 underwent remission, followed by subsequent relapse. Three patients had a third relapse. The time interval between primary surgical treatment and patient death confirmed to be attributable to complications of ovarian carcinoma–the overall survival time–ranged from 3 to 79 months and had a mean and a median of 23 and 20 months, respectively. Patient deaths attributable to other causes were considered censored events. Of the patients remaining alive at the termination of the study in April 1998, recurrent disease or metastasis was identified in 22 patients (29%) but was undetectable in 54 patients (71%).

Patients were characterized for a number of clinicopathological variables at the time of surgery (Table 1). These included stage classified according to the FIGO (36), which required that

[3] The abbreviations used are: DFS, disease-free survival; OS, overall survival; FIGO, International Federation of Gynecology and Obstetrics; RR, relative risk; CI, confidence interval.

Table 1 Patient characteristics

	Number (%)
Stage (FIGO)	
I	17 (14)
II	7 (6)
III	88 (73)
IV	8 (7)
Grade (WHO)	
1	13 (11)
2	31 (26)
3	76 (63)
Histologic type	
Serous papillary	47 (39)
Endometrioid	20 (17)
Clear cell	18 (15)
Mucinous	8 (7)
Mullerian	6 (5)
Undifferentiated	21 (17)
Debulking success	
Optimal	50 (42)
Suboptimal	70 (58)
Residual tumor (cm)	
≤1	48 (40)
1–3	33 (27)
4–8	26 (22)
≥9	13 (11)

extensive surgical and cytological assessment of the disease extent was performed in all cases. These procedures included collection of ascites or peritoneal washings from the pelvis, gutters, and diaphragms for cytological studies; total abdominal hysterectomy and bilateral salpingo-oophorectomy; infracolic omentectomy and appendectomy; selective pelvic and paraaortic lymphadenectomy; and debulking of all gross tissues. 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. Histological grade and type based on WHO criteria (37), as well as other clinicopathological variables, are also summarized in Table 1.

All patients had been previously untreated for ovarian cancer. According to standard practice (38), administered as first-line chemotherapy to the majority of patients were combinations of chemotherapeutic agents including cisplatin together with cyclophosphamide alone ($n = 32$) or in addition to either doxorubicin ($n = 10$) or epirubicin ($n = 12$). Cisplatin was also administered together with either paclitaxel ($n = 6$), epirubicin ($n = 2$), or epirubicin plus methotrexate ($n = 1$). Carboplatin was given together with either cyclophosphamide ($n = 8$), or paclitaxel alone ($n = 14$) or in addition to epirubicin ($n = 4$) or doxorubicin ($n = 1$). Cisplatin and carboplatin alone were given to 13 and 17 patients, respectively. All patients had received either cisplatin or carboplatin. Three patients additionally received radiotherapy, and another two were given hormonotherapy. Assessment of treatment response, by computed tomography scanning, serum CA125 determination and, in some cases, by second-look laparotomy, in the 72 patients with residual tumor size >1 cm was performed after the last cycle of chemotherapy and was based on the following criteria (39): resolution of all evidence of disease for at least 1 month was considered a complete response; a decrease of ≥50% in the product of the diameters (maximal and minimal) of all measurable lesions lasting at least 1 month without the development of new lesions was considered a partial response; a decrease of <50% or an increase of <25% in the product of the diameters of all measurable lesions was considered stable disease; and an increase of ≥25% in the measurable lesions as described above or the identification of new lesions was considered progressive disease. The majority of patients initially responded completely ($n = 36$) or partially ($n = 22$) to first-line chemotherapy, whereas others experienced no change ($n = 7$) or progressive disease ($n = 7$). Second-line chemotherapy after initial treatment failure was given to 67 patients and included cisplatin ($n = 6$), carboplatin ($n = 12$), cyclophosphamide ($n = 9$), paclitaxel ($n = 25$), epirubicin ($n = 11$), and doxorubicin ($n = 4$). Twelve patients required third-line chemotherapy, consisting of cisplatin ($n = 3$), carboplatin ($n = 1$), cyclophosphamide ($n = 1$), and paclitaxel ($n = 7$).

Tumor Extraction. Tumor tissues were snap-frozen in liquid nitrogen immediately after surgery according to a standard protocol for the preparation of frozen sections, histological examination of which allowed representative portions of each tumor containing >70% tumor cells to be selected for storage at −80°C until analysis. A subset of randomly selected tumors ($n = 27$) were sampled at two different surfaces to yield portions that were separately pulverized, extracted, and assayed as described below. Approximately 200–300 mg of each specimen was pulverized to a fine powder on dry ice and combined with 1 ml of a cell lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 10 ml/l NP40 surfactant, 10 mg/l phenylmethylsulfonyl fluoride, and 1 mg/l each of aprotinin and leupeptin) for 30 min on ice before centrifugation at 14,000 × *g* for 30 min at 4°C to collect the supernatants. The crude lysates were assayed immediately and concurrently for p53 protein by immunofluorometry, p21 protein by colorometric immunoassay, and total protein content by a kit based on the bicinchoninic acid method (Pierce Chemical, Rockford, IL).

Immunoassay of p53 Protein. Concentrations of soluble p53 protein in the ovarian tumor extracts were determined without knowledge of the corresponding patient clinicopathological or survival information by a quantitative, sandwich-type immunoassay described in detail elsewhere (40). This method used the ability of p53 protein to react simultaneously with two different immunoreagents, mouse monoclonal DO-1 antibody (gift of Dr. David Lane, University of Dundee, Dundee, United Kingdom) immobilized onto microtiter wells before sample addition, and subsequently added rabbit polyclonal CM-1 antiserum (Novocastra, Newcastle upon Tyne, United Kingdom). Bound p53-antibody complexes were detected after sequential additions of alkaline phosphatase-conjugated goat antirabbit immunoglobulin (Jackson ImmunoResearch, West Grove, PA), the enzyme substrate diflunisal phosphate, and finally a Tb^{3+}-EDTA chelate with which the dephosphorylated reaction product can complex at alkaline pH. In a dedicated instrument [Cyberfluor-615 Immunoanalyzer (Cyberfluor, Toronto, Ontario, Canada)], the fluorescence emitted from the final solution at 615 nm after excitation at 336 nm was measured, both in the wells corresponding to the unknown samples and in those to

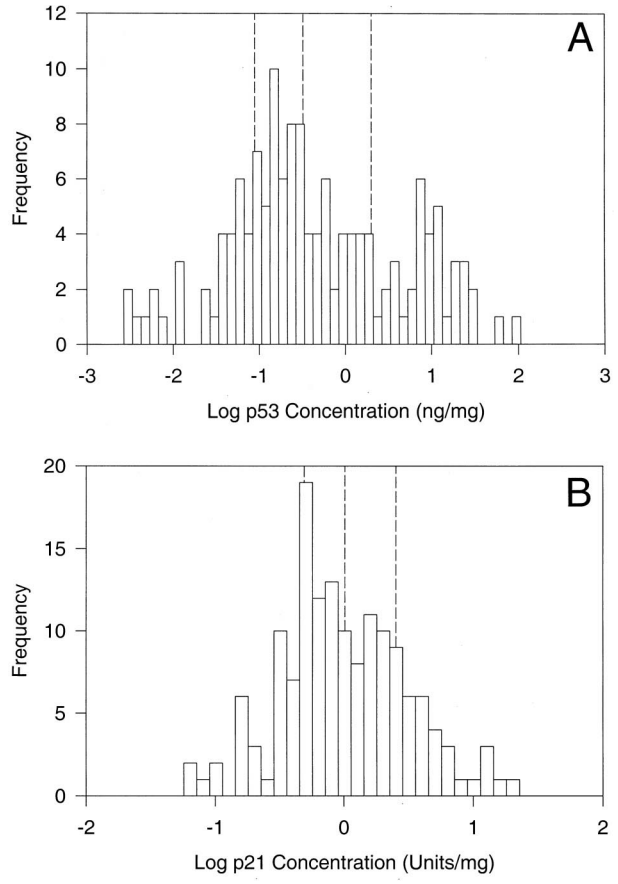

Fig. 1 Frequency distributions of logarithmically transformed p53 (*A*) and p21 (*B*) concentrations in the 118 (of 120) and 118 (of 118) ovarian tumor extracts, respectively, that had p53 and p21 levels exceeding the assay detection limits. From *left* to *right*, the *dashed lines* indicate the 25th, 50th, and 75th percentiles of each distribution.

Table 2 p53 concentrations[a] in relation to other clinicopathological variables

Factor	Number	Median	Range	P^b
p21 (units/mg)				
<median	61	0.42	0–102.51	
≥median	57	0.30	0–66.61	0.54
Age (yr)				
<55	57	0.41	0–102.51	
≥55	63	0.23	0–34.34	0.36
Stage				
I–II	24	0.21	0–12.16	
III–IV	96	0.60	0–102.51	0.02
Grade				
1	13	0.20	0–1.73	
2	31	0.33	0–102.51	
3	76	0.60	0–66.61	0.03
Histological type				
Serous	47	0.68	0–102.51	
Others	73	0.31	0–34.34	0.14
Residual tumor (cm)				
<1	48	0.25	0–66.61	
≥1	72	0.60	0–102.51	0.02
Patient relapse				
No	54	0.23	0–66.61	
Yes	66	0.63	0–102.51	0.04
Patient death				
No	76	0.19	0–102.51	
Yes	44	1.20	0–29.07	<0.01

[a] p53 concentrations expressed in ng/mg.
[b] *P* determined from Wilcoxon rank sum tests with continuity correction or Kruskal-Wallis tests, where appropriate.

which the assay calibrators, assayed in parallel, had been added. Both unknowns and calibrators were assayed in duplicate. The calibrator solutions, ranging in concentration from 0.15 to 75 ng/ml, were prepared by dilutions of a lysate of Sf9 cells infected with a p53-expressing recombinant baculovirus (gift of Dr. Thierry Soussi, Institut Curie, Paris, France), as described previously (41). Concentrations of p53 protein exceeding the detection limit of ~0.04 ng/ml were divided by the total protein contents of the extracts to adjust for differences in tissue masses and extraction efficiencies.

Immunoassay of p21 Protein. The WAF1 Quantitative ELISA Assay (Oncogene Research, Cambridge, MA) was used to measure p21 concentrations in the ovarian tumor extracts, following the manufacturer's instructions. All necessary reagents were provided in the kits. Features of this sandwich-type immunoassay include a rabbit polyclonal anti-p21 antibody immobilized onto microtiter plates, a biotinylated mouse monoclonal antibody specific for human p21 protein added after sample addition, and detection by streptavidin conjugated to horseradish peroxidase, which catalyzes the conversion of tetramethylbenzidine into a colored product. Dual wavelength absorbances at 450 and 540 nm were determined using a microplate spectrophotometer (Labsystems, Helsinki, Finland). Using dedicated software, concentrations of p21 were interpolated from calibration curves constructed from the assay of lyophilized p21 standards ranging in concentration from 0 to 20 units/ml. Calibrators and ovarian tumor extracts were assayed in duplicate and in parallel. Concentrations of p21 greater than the reported lower limit of detection of 0.1 unit/ml were expressed as units/mg, adjusting for the variable protein contents of the extracts. Extracts prepared from breast carcinoma cells (MCF-7 and T-47D), obtained from the American Type Culture Collection, cultured as described elsewhere (40), and for which the p21 expression status had been characterized previously (41), were assayed in parallel as qualitative positive and negative controls, respectively.

Statistical Analysis. The statistical analysis, performed using SAS version 6.12 software (SAS Institute, Cary, NC), examined associations between the total protein-adjusted p53 and p21 immunoassay results and DFS and OS, as well as between the p53 and p21 concentrations and other measurements or characteristics of the sample of ovarian cancer patients. All procedures were nonparametric and based on two-tailed tests of significance. Monotonic relationships between p53 and p21 as continuous variables were shown by the calculation of the Spearman correlation coefficient. Continuity-corrected Wilcoxon rank sum tests or Kruskal-Wallis tests were used to compare the distributions of p53 and p21 concentrations, one at a time, between patient subgroups defined by their status for the other protein marker (p21 or p53, each classified as negative or

Table 3 p21 concentrations[a] in relation to other clinicopathological variables

Factor	Number	Median	Range	P^b
p53 (ng/mg)				
<median	58	1.14	0.09–24.54	
≥median	60	0.94	0.11–17.10	0.68
Age (yr)				
<55	61	1.17	0.09–24.54	
≥55	57	0.82	0.07–13.25	0.17
Stage				
I–II	24	1.65	0.09–24.54	
III–IV	94	0.83	0.07–10.49	0.13
Grade				
1	13	1.84	0.09–24.54	
2	30	0.77	0.09–17.10	
3	75	0.88	0.07–8.78	0.22
Histological type				
Serous	46	0.82	0.16–13.26	
Others	72	1.11	0.09–24.54	0.42
Residual tumor (cm)				
<1	47	1.23	0.09–24.54	
≥1	71	0.81	0.07–8.78	0.13
Patient relapse				
No	54	0.96	0.09–24.54	
Yes	64	0.78	0.07–15.62	0.21
Patient death				
No	74	1.17	0.09–24.54	
Yes	44	0.76	0.07–15.62	0.05

[a] p21 concentrations expressed in units/mg.
[b] *P* determined from Wilcoxon rank sum tests with continuity correction or Kruskal-Wallis tests, where appropriate.

positive using cutoff points equal to the 50th percentiles of the respective distributions) and for each of the clinicopathological variables: age (<55 years *versus* >55 years), FIGO stage (stages I or II *versus* stages III or IV), histological grade (grade 1 *versus* grade 2 *versus* grade 3), histological type (serous papillary *versus* all other histotypes), and residual tumor size (<1 cm *versus* >1 cm). Differences in p53 and p21 expression status, as well as differences in patient age, tumor grade, and histological type classified as above, between patients with assessable postoperative disease who exhibited either complete response to first-line chemotherapy, partial response to such treatment, stable disease, or progressive disease despite having received first-line chemotherapy were determined by two-tailed Fisher exact tests. Wilcoxon rank sum tests were also used to examine the occurrences, during follow-up, of ovarian cancer relapse and patient death in relation to p53 and p21 concentrations.

The relationships of p53, p21, and other clinicopathological variables to DFS and OS were evaluated by the RRs for relapse and death and their 95% CIs, which were calculated from fitted Cox proportional hazards regression models. In the multivariate analysis, the models were adjusted for age, stage, grade, and residual tumor size, all of which were considered dichotomous or three-level variables defined by the classification criteria given above. In both univariate and multivariate models, p53 was examined separately as a dichotomous variable categorized by the median percentile cutoff point and as a quartile-divided, four-level ordinal variable. The prognostic value of median-dichotomized p21 was determined by fitting a univariate Cox model. To determine the prognostic impact of p53 and p21 assessed in combination, a three-level ordinal variable was created and evaluated in Cox models of DFS and OS. The first level of this new variable included patients whose tumor extracts were concurrently p53 negative and p21 positive. The second level consisted of patients whose tumors were either positive for both markers or negative for both markers. Patients in the third level had tumors that were p53 positive and p21 negative. Kaplan-Meier survival curves were also constructed to demonstrate the effects of p53 concentrations exceeding the median percentile on DFS and OS probabilities, differences over time that were evaluated using log-rank tests. The same Kaplan-Meier analyses were performed to reveal differences in survival between p21-negative and p21-positive patients.

RESULTS

Distributions of p53 and p21 Concentrations. Of the 120 ovarian tumor extracts, all except 2 had detectable p53 protein concentrations. When adjusted for the total protein contents of the extracts, these concentrations ranged from 0.005 to 102.51 ng/mg and were bimodally distributed with a mean of 5.24 ng/mg, an SD of 12.76 ng/mg, and 25th, 50th, and 75th percentiles of 0.10, 0.42, and 5.05 ng/mg, respectively (Fig. 1*A*). The high degree of concordance (r_s = 0.87; P = 0.0001) between p53 concentrations measured in 27 pairs of extracts prepared from two different portions of the same tumors suggested that p53 accumulation throughout each tumor specimen used for analysis was roughly homogeneous. p21 concentrations in the 27 pairs of extracts were also correlated (r_s= 0.63; P = 0.006), but indicated that p21 exhibited greater intratumor variability. In the extracts of all 118 tumors assayed for p21, the concentrations of this analyte exceeded the lower detection limit of the assay in all cases. Adjustment of these values for the total protein contents of the extracts yielded a distribution that ranged from 0.07 to 24.54 units/mg and had a mean, SD, and median of 1.93 units/mg, 3.08 units/mg, and 0.82 units/mg, respectively (Fig. 1*B*). The 25th and 75th percentiles of the p21 distribution were 0.52 units/mg and 2.08 units/mg, respectively.

Relationships between p53, p21, and Other Clinicopathological Variables. Given the ability of functional, nonmutant p53 to induce p21 expression and to identify possible interaction between the two proteins in survival analysis, it was of interest to determine whether the bulk tumor tissue concentrations of p53 and p21 were associated with each other. Concentrations of p53 and p21 were not correlated (r_s = 0.07; P = 0.46). Moreover, neither the difference in p53 concentrations between median-dichotomized p21-negative and p21-positive specimens (Table 2), nor the difference in p21 concentrations between tumor extracts classified as p53 negative and p53 positive using the median p53 value (Table 3), were statistically significant by Wilcoxon rank sum tests. Using the same analysis, associations between each of these proteins and the status of the other clinicopathological features for which the ovarian tumors were characterized were also examined because of possible confounding influences of these other variables upon the relationships between patient survival times and p53 or p21 concentrations. Table 2 shows that although p53 concentrations did not differ significantly between the two groups of patients who were younger and older, respectively, than the median age,

Table 4 Associations between clinicopathological variables and response to adjuvant chemotherapy

Factor	Number responding (%)				P^a
	Complete response	Partial response	Stable disease	Progressive disease	
p53 status					
<median	20 (63)	10 (31)	2 (6)	0 (0)	
≥median	16 (40)	12 (30)	5 (13)	7 (17)	0.03
p21 status					
<median	19 (54)	11 (31)	3 (9)	2 (6)	
≥median	17 (46)	11 (30)	4 (11)	5 (13)	0.76
Age (years)					
<55	16 (52)	11 (35)	3 (10)	1 (3)	
≥55	20 (49)	11 (27)	4 (10)	6 (14)	0.48
Grade					
1	2 (100)	0 (0)	0 (0)	0 (0)	
2	12 (80)	2 (13)	1 (7)	0 (0)	
3	22 (40)	20 (36)	6 (11)	7 (13)	0.11
Histological type					
Serous	13 (38)	15 (44)	3 (9)	3 (9)	
Others	23 (61)	7 (19)	4 (10)	4 (10)	0.12

[a] *P* determined from two-tailed Fisher exact tests.

concentrations of p53 were higher in extracts prepared from ovarian cancers that were more advanced (stages III–IV), less well-differentiated (grade 3), and suboptimally debulked (residual tumor diameter >1 cm). A trend suggesting that serous ovarian carcinomas may have had higher p53 concentrations than all other histological types was also revealed. As shown in Table 3, none of these other clinicopathological variables was significantly associated with p21 concentrations by Wilcoxon rank sum analysis.

Relationships between Each Clinicopathological Variable and Patient Response to Treatment. The assessment of clinical response to platinum-based adjuvant treatment of 72 patients with measurable (>1 cm) postoperative lesions enabled comparison of the distributions of p53-negative and p53-positive specimens between patients who exhibited complete response to chemotherapy, partial response, stable disease, or progressive disease. Table 4 presents this comparison, which demonstrated that tumor extracts containing p53 protein at levels exceeding the median concentration were more frequently obtained from patients who did not respond to treatment. In contrast, p21 positivity status, patient age group, histological grade, and histological type were statistically unrelated to the classification of patients into treatment response groups. The relationship between disease stage and response could not be statistically evaluated because all patients in stages I or II had complete response to adjuvant chemotherapy (data not shown).

p53 and p21 as Indicators of Ovarian Cancer Survival. Several approaches, including comparisons of Kaplan-Meier survival plots and fitting of Cox proportional hazards regression models, were used to show associations between patient postoperative prognosis and concentrations of p53 and p21, considered individually, in combination with each other, and jointly with the other prognostic factors. A relationship between p53 and patient survival had already been suggested by findings that p53 concentrations were higher in tumor extracts from patients who relapsed or died during their follow-up periods (Table 2). The similar analysis for p21, shown in Table 3, revealed reduced levels of p21 in tumors of patients who died of ovarian cancer. Consistent with these preliminary results with respect to p53 were the findings of regression analysis, by which the RRs for both relapse and death were shown to be significantly increased for p53-positive patients when p53 was classified into two groups based on the median (Table 5). The use of the median cutoff for p53 also indicated 50% and >90% increased risks for relapse and death, respectively, of p53-positive patients in multivariate analysis adjusted for all of the other variables except histological type. Furthermore, by classifying patients into four groups based on the quartiles of the p53 distribution, statistically significant trends were demonstrated, possibly implying that successively increasing levels of p53 were associated with successively increasing risks for both relapse and death. However, comparisons of risks for relapse and death between patients in the first quartile to those in the second, third, and fourth quartiles did not reveal significant differences, as shown by the overlapping confidence intervals. In the corresponding multivariate models, the dose-response relationships suggested by the univariate analyses for trends did not reach statistical significance. The differences in the survival rates over time between p53-negative and p53-positive patients are shown in Fig. 2. Whereas these results established p53 to be an independent prognostic factor in our series of ovarian cancer patients, both univariate Cox regression (Table 5) and Kaplan-Meier analysis (Fig. 3) revealed that p21 negativity based on a median cutoff value was not associated with relapse and death rates. Use of either the 25th or 75th percentiles as cutoff points for defining p21 positivity similarly did not lead to significant differences in DFS or OS between p21-negative and p21-positive patients (data not shown). On the other hand, because there was evidence of a trend for median-dichotomized p21-negative patients to have increased risk for death, and given the prognostic value of median-dichotomized p53, a composite three-level variable was created and evaluated in the Cox regression analysis. Patients in the first category, expected to have the best prognosis, were defined as having tumors that were p53 negative and p21 positive. Patients who had either p53-positive, p21-positive tumors or p53-negative, p21-negative tumors were members of

Table 5 Associations between p53 and p21 concentrations and DFS and OS

p53 or p21 status	Disease-free survival			Overall survival		
	RR[a]	95% CI[b]	*P*	RR[a]	95% CI[b]	*P*
Univariate analysis of p53 ($n = 120$)						
Based on median cutoff point						
Negative	1.00			1.00		
Positive	1.64	1.01–2.67	0.04	2.75	1.41–5.32	<0.01
Based on quartiles[c]						
First quartile	1.00			1.00		
Second quartile	0.66	0.31–1.41		0.77	0.25–2.45	
Third quartile	1.10	0.72–1.44		1.43	0.89–2.30	
Fourth quartile	1.28	0.96–1.47		1.51	1.06–1.97	
P for trend			0.04			<0.01
Univariate analysis of p21 ($n = 118$)						
Based on median cutoff point						
Positive	1.00			1.00		
Negative	1.14	0.68–1.78	0.56	1.35	0.75–2.44	0.17
Univariate analysis of p53-p21 ($n = 118$)[d]						
p53(−), p21(+)	1.00			1.00		
p53(+), p21(+) or p53(−), p21(−)	1.35	0.76–1.93		1.16	0.52–2.57	
p53(+), p21(−)	1.44	0.96–2.73		1.38	0.92–2.08	
P for trend			0.07			0.06
Multivariate analysis of p53 ($n = 120$)[e]						
Based on median cutoff point						
Negative	1.00			1.00		
Positive	1.50	0.63–2.17	0.05	1.92	0.93–3.96	0.03
Based on quartiles[c]						
First quartile	1.00			1.00		
Second quartile	0.73	0.31–1.72		0.69	0.11–4.30	
Third quartile	1.12	0.76–1.46		1.52	0.94–3.16	
Fourth quartile	1.31	0.98–2.75		1.77	0.99–3.35	
P for trend			0.09			0.07

[a] RR estimated by the Cox proportional hazards regression model.
[b] 95% CI of the estimated RR.
[c] Estimated RR for second, third, and fourth quartiles compared with the first quartile are given. *P*s are based on 1 degree of freedom tests of monotonic association.
[d] p53 positivity and p21 positivity based on median cutoff points. Estimated RR for second and third groups compared with first group are given. *P*s are based on 1 degree of freedom tests of monotonic association.
[e] Multivariate analysis adjusted for age, stage, grade, and residual tumor size.

the second group. Having the anticipated worst prognosis were patients in the third group, whose tumor extracts were p53-positive and p21-negative. As shown in Table 5, although the *P* for trends were of borderline significance, this analysis suggested that the combination of increasing p53 concentrations and decreasing p21 concentrations was associated with higher risks for relapse and death. In addition to p53 positivity, other clinicopathological features indicative of poor prognosis in multivariate Cox models were late-stage (II–IV) malignancy, associated with a RR for relapse of 9.08 (95% CI, 3.89–21.20; $P < 0.01$) and a RR for death of 33.44 (95% CI, 4.59–243.43; $P < 0.01$), poorly differentiated (grade 3) tumors, associated with RRs for relapse and death of 9.14 (95% CI, 2.87–29.11; $P < 0.01$) and 16.01 (95% CI, 2.21–116.56; $P < 0.01$), respectively, and residual tumor size >1 cm, associated with RRs of 11.25 (95% CI, 5.89–21.51; $P < 0.01$) and 23.30 (95% CI, 7.20–75.38; $P < 0.01$) for relapse and death, respectively.

DISCUSSION

The majority of patients treated surgically for epithelial ovarian cancer subsequently receive systemic therapy, most often with platinum-containing antineoplastic regimens although paclitaxel, cyclophosphamide, or other agents are also used individually or as polychemotherapy. Resistance to these drugs may be reflected, in part, by higher rates of relapse and death and is thought to be multifactorial in origin (42). Several molecular factors likely contributing to loss of chemosensitivity in ovarian carcinoma have been identified, including proteins mediating the transport and cellular turnover of drugs as well as those involved in DNA repair and other nonspecific defense mechanisms. It has become apparent that conventional chemotherapeutic agents exert their function ultimately via the cellular machinery governing cell cycle progression and programmed cell death, and that the pathways regulating these processes are fundamentally perturbed in cancer cells (43). Playing a central role in both processes is p53, alterations of which are strongly associated with chemoresistance and radioresistance in hematological malignancies (44). In the majority of solid tumors, however, a correlation between p53 mutation and prognosis or chemotherapy response has not been consistently demonstrated. For instance, although a number of studies have shown an association between p53 alteration and poor prognosis of ovarian cancer patients (24, 25), other studies have contradicted these findings (26, 27). Similarly, evidence implicating the

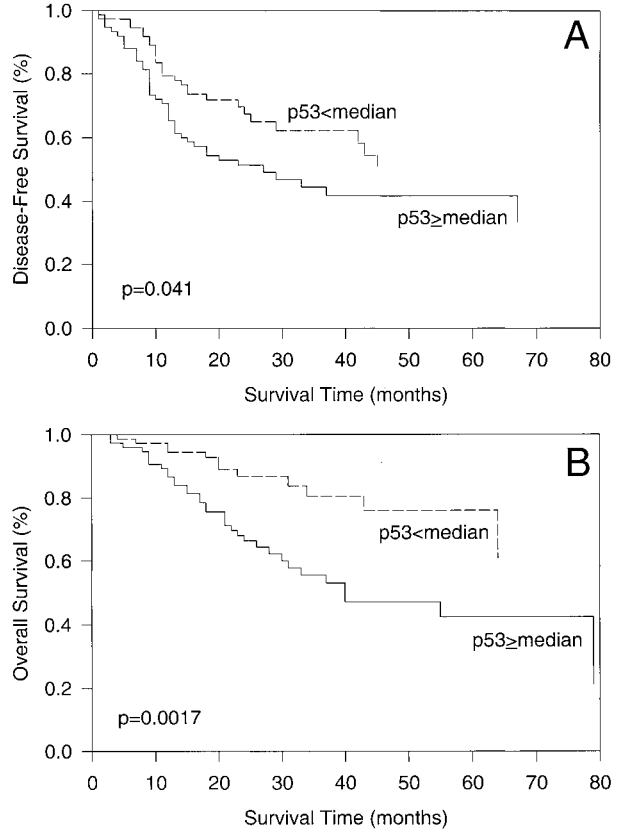

Fig. 2 Kaplan-Meier analysis of DFS (*A*) and OS (*B*) of the 120 ovarian cancer patients treated with first-line chemotherapy, using the median p53 concentration as the cutoff point for p53 positivity. *P*s were determined by log-rank tests.

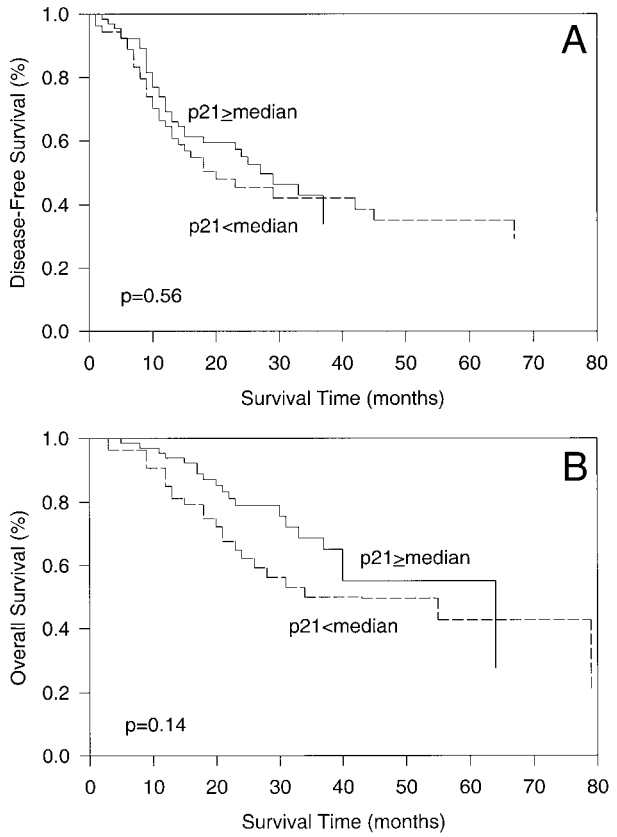

Fig. 3 Kaplan-Meier analysis of DFS (*A*) and OS (*B*) of the 118 ovarian cancer patients treated with first-line chemotherapy, using the median p21 concentration as the cutoff point for p21 positivity. *P*s were determined by log-rank tests.

involvement of p53 in resistance of ovarian neoplasms to chemotherapy, provided primarily by the detection of mutations or deletions in the *p53* gene in chemoresistant human ovarian cancer cell lines (45–47), has been accompanied by other reports showing that chemotherapy-induced apoptosis may occur in the absence of functional p53 (48, 49) and that cisplatin resistance may develop independently of p53 alterations (50, 51). Clinical studies of the effect of *p53* gene status on the response of ovarian cancer patients to cisplatin-based adjuvant chemotherapy have also emerged and have yielded findings suggestive of a role for p53 as a determinant of chemosensitivity (52, 53). The effect of cisplatin-paclitaxel combination treatment for advanced ovarian cancer, on the other hand, was shown not to be influenced by p53 mutation in another study (54). To the best of our knowledge, none of these clinical studies has additionally assessed the expression of the p21 protein, high levels of which have paradoxically been associated with chemoresistance in acute myelogenous leukemia patients (55). Because p21 has been shown to be induced by cisplatin in both chemosensitive and chemoresistant human ovarian carcinoma cell lines (56) and to be not absolutely correlated with p53 expression levels in normal and malignant ovarian epithelial cells (57), it remained possible that p21 expression in ovarian tumors might predict cisplatin responsiveness independently of p53 expression. Considering this possibility, we studied the prognostic and predictive implications of both p53 and p21 expression levels in epithelial ovarian cancer.

Quantitative immunoassays were used to determine the expression levels of p53 and p21 in 120 tumors from patients treated with platinum-based adjuvant chemotherapy. For each protein studied, a continuous distribution of concentrations was revealed to be present in the tumor extracts. The concentrations of p53 and p21 in extracts of the matched normal ovarian tissues, however, were unknown, thus precluding the categorization of patients as p53-positive or p21-positive in cases where levels of these markers exceeded upper limits of the normal ranges of values. Alternative, objective cutoff points for defining p53 and p21 positivity were the medians of the respective distributions. The immunofluorometric procedure used to assay p53 levels in these extracts was developed in our laboratory (40) and has been validated by comparison of its findings to p53 immunostaining of matched formalin-fixed, paraffin-embedded lung tumors (35) and to sequencing of exons 5 to 9 of the *p53* gene in a series of 55 ovarian carcinomas (58). In the latter study, 10 of the 12 identified missense mutations were accompanied by p53 concentrations exceeding the 75th percentile, and tumors with nonsense and frameshift mutations were invariably

p53 negative based on this cutpoint. However, 5 of 39 tumors without mutations were also considered p53 positive, further indicating the imperfect concordance between the two methods for the detection of p53 abnormalities. Despite the differences, both the results of p53 immunoassay and of mutational analysis demonstrated significant associations between p53 and advanced disease (58). Although our assay procedure has not been directly compared with immunohistochemistry performed on ovarian carcinomas, it might possess several advantages. In principle, because of the rigorous washing steps, effective immunopurification of antigen from background signal-eliciting tissues and the use of two p53-specific antibodies rather than the single primary antibody used for immunostaining, a sandwich-type immunoassay for p53 would be expected to have greater analytical specificity than conventional immunohistochemical methods of p53 detection. Moreover, the results of a quantitative immunoassay are inherently more objective because they can be evaluated by numerical decision thresholds, simplifying the statistical analysis and quality control. The chief disadvantages of a p53 immunoassay relative to immunostaining, however, are the requirement for fresh frozen tissues and the loss of information relating p53 expression to cellular or histological features. Relative to the analysis of p53 mutation at the genetic level, an immunoassay for p53 protein suffers from the same major disadvantage as immunostaining methods, *i.e.,* the imperfect concordance between p53 mutation and p53 protein accumulation. Notwithstanding these limitations, in this study, comparisons of p53 concentrations between patients with different pathological features, treatment responses as defined by standard criteria, and risks for relapse and death estimated by Cox regression analysis demonstrated significantly increased p53 concentrations in tissues from patients with more aggressive, treatment-refractory ovarian cancers. Comparisons of p21 concentrations between the same groups of patients did not reveal significant differences, suggesting that tumor tissue levels of this protein may not have been deterministic of prognosis or chemotherapy response in the patients studied. Our findings are concordant with those of other groups reporting the independent prognostic value of p53 protein expression in ovarian carcinoma (25, 28), as well as with our own previous study of a smaller sample of epithelial ovarian cancer patients for whom details of the chemotherapy regimens and responses were unavailable (24). Our results also complement the small number of recent studies that have suggested a correlative relationship between p53 alterations and clinical response of ovarian cancer to chemotherapeutic agents (52, 53). However, our other findings that neither the assignment of treatment response category nor the probability of DFS or OS were shown to be affected by the p21 levels in the ovarian tumor extracts are novel but consistent with the lack of an absolute negative correlation between p21 and p53 expression levels found here and elsewhere (41, 57), as well as with *in vitro* observations that anticancer drug sensitivity is not always dependent on p21 expression (56). Also novel, in our opinion, is the detection of p21 protein in ovarian tumor extracts by an immunoassay instead of immunostaining. Although the two procedures were not performed in parallel to validate the results of the commercially developed p21 immunoassay, our confidence in the latter's results, at least qualitatively, was provided from the assay of extracts prepared from cell lines for which the expression status of p21 was already known.

The relationship between the p53 overexpression status of primary ovarian carcinoma specimens obtained at surgery and the subsequent designation of response to first-line chemotherapy was examined in a subset of patients. Although our results suggest that patients with elevated p53, arbitrarily defined as having p53 concentrations exceeding the median value, were more likely to exhibit treatment failure, they must be interpreted cautiously. Because the majority of patients received cytotoxic agents in addition to cisplatin or carboplatin, it remains possible that the effects of these other drugs may have modulated the treatment responses independently of p53 status. Moreover, our demonstration in multivariate regression analysis adjusted for stage, residual tumor presence, age, and histotype that p53 was an independent prognostic factor in our sample of chemotherapy-treated patients does not necessarily lead to the conclusion that p53 is predictive of treatment response. Over half of the patients in our series received second-line chemotherapy with various agents that might have contributed to relapse-free survival and OS. For these reasons, the results of our investigation must be confirmed by other studies of epithelial ovarian cancer patients receiving single-agent therapy.

In summary, the quantitative analysis of p53 and p21 proteins in extracts of ovarian carcinomas confirmed the prognostic value of p53 and provided evidence that p53 protein accumulation may predict responsiveness to postoperative chemotherapy. The assessment of p21 expression in ovarian cancer, however, was shown to be of questionable clinical value. Despite these latter observations, future studies of larger numbers of ovarian carcinoma patients with more restricted treatment regimens might clarify the prognostic and predictive values of p21 and p53 in combination.

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