

p27^{Kip1} Expression Is Associated with Clinical Outcome in Advanced Epithelial Ovarian Cancer: Multivariate Analysis¹

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ABSTRACT

Few biological parameters have been shown to have a prognostic role in patients with advanced ovarian cancer. p27^{Kip1} is a cyclin-dependent kinase inhibitor, and its loss may contribute to tumor progression. We determined whether p27^{Kip1} protein expression in advanced ovarian cancer could be associated with prognosis. p27^{Kip1} status was assessed by immunohistochemical analysis of tissue sections from primary tumors of 99 patients with stages III–IV ovarian carcinoma and was analyzed in relation to clinicopathological variables, time to progression (TTP), and overall survival (OS). p27^{Kip1} expression was detected in 47 (47%) of the 99 patients. p27 expression did not correlate with any of the classical clinicopathological parameters. Loss of p27 protein was significantly associated with a short TTP ($P = 0.0004$) and decreased OS ($P = 0.0302$). The 5-year TTP rate in p27-positive patients was 50% versus 11% in p27-negative patients. p27-positive cases showed a 5-year OS rate of 53% compared with 43% of p27-negative cases. In multivariate analysis, p27 expression was an independent predictor of progression of disease ($P = 0.0009$) and survival ($P = 0.0032$) when considered together with stage of disease, presence of ascites, and residual tumor at surgery.

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Loss of p27^{Kip1} conferred poor prognosis independently of proliferative index, as assessed by proliferating cell nuclear antigen immunostaining. p27 immunoreactivity can be used to predict progression of disease and survival in patients with advanced epithelial ovarian cancer and therefore may represent a new prognostic marker.

INTRODUCTION

Ovarian cancer is the most common gynecological malignancy causing fatality in Western countries and constitutes the fifth leading cause of female cancer death (1). Over 70% of women present with an advanced stage of disease at the time of diagnosis, and despite cytoreductive surgery and the effectiveness of platinum-based chemotherapy, tumors progress in most patients, becoming resistant to chemotherapy (2). Traditional clinicopathological criteria used to predict clinical outcome are largely inadequate. Thus, the characterization of biological prognostic factors that may help in identifying patients with different clinical outcomes would greatly facilitate management of disease. The *p53* tumor suppressor gene has been suggested to play an important role in influencing tumor cell sensitivity to chemotherapy in “*in vitro*” models as well as in clinical settings (3, 4).

Recently, much attention has been focused on the potential prognostic role of cell cycle inhibitors in ovarian cancer. In particular, p27^{Kip1} is a cdk³ inhibitor that regulates progression from G₁ into S-phase by inhibiting a variety of cyclin-cdk complexes, including cyclin D-cdk4, cyclin E-cdk2, and cyclin A-cdk2 (5). The *p27^{Kip1}* gene is located on chromosome 12p and, unlike the genes encoding INK4 family members, is rarely affected by structural alterations in human cancer (6).

Several studies have demonstrated that loss of p27^{Kip1} protein is associated with progression in various malignancies (7–12). In ovarian cancer, we and others (13–15) have shown previously that loss of p27 protein is a frequent event in primary epithelial ovarian carcinomas but not in benign and low malignant potential ovarian tumors. However, conflicting data about the possible prognostic role of p27 status in advanced ovarian cancer patients have been reported (16, 17).

The objective of this study was to examine the value of p27^{Kip1} protein in predicting prognosis in a large cohort of uniformly treated patients with stages III–IV epithelial ovarian cancer, followed up over a long period of time. The correlation with response to chemotherapy, PCNA, and p53 status was also investigated.

³ The abbreviations used are: cdk, cyclin-dependent kinase; PCNA, proliferating cell nuclear antigen; TTP, time to progression; OS, overall survival; CI, confidence interval; RR, relative risk.

Table 1 Distribution of p27-positive and p27-negative cases according to patient characteristics

	p27 positive	p27 negative	P
Histology			
Serous	31	36	NS ^a
Mucinous	1	1	
Endometrioid	9	5	
Undifferentiated	4	8	
Other	2	2	
Stage			
III	41	40	NS
IV	6	12	
Grading			
1-2	8	6	NS
3	37	43	
NA	2	37	
Ascites			
No	18	19	NS
Yes	29	33	
Residual tumor			
≤2 cm	32	26	NS
>2 cm	15	26	
Response to chemotherapy			
CR/PR	41	36	0.089
NC-P	4	11	
NA	2	5	
p53 status			
Negative	18	22	NS
Positive	27	27	
PCNA status			
Negative	31	17	NS
Positive	31	20	
Age (yr)			
≤60	31	34	NS
>60	16	18	

^a NS, not significant; NA, not available; CR, complete response; PR, partial response; NC-P, no change-progression.

MATERIALS AND METHODS

Patients. The study included 99 primary advanced epithelial ovarian cancer patients who underwent surgical resection in the Department of Gynecology of the Catholic University of Rome. Patients had stage III/IV disease according to the Fédération Internationale des Gynaecologistes et Obstétristes staging system. Histological classification of tumors was carried out according to the WHO system (18), and tumors were graded as well (G1), moderately (G2), and poorly differentiated (G3). Although several cutoff values of residual volume tumor have been proposed, it has been reported that gradual gradations of residual disease can affect ovarian cancer prognosis (19). Our patient population was divided into two groups according to the extent of residual disease at first surgery; 58 patients had ≤2 cm residual disease, whereas 41 patients had >2 cm residual disease at surgery. Patient characteristics, collected by retrospective chart reviews, are shown in Table 1. Chemotherapy was instituted 2-3 weeks after surgery. All patients received four to six cycles of cisplatin-containing regimens (total cisplatin dose, at least 400 mg/m²). Gynecological examination, abdominal pelvic ultrasonography, CA-125 assay, and radiological investigations, if necessary, were performed monthly for

the clinical assessment of response, which was recorded according to WHO criteria (18). Approximately 28 days after the last course, clinical complete responders underwent second-look laparoscopy. In laparoscopy-negative cases, second-look laparotomy was performed for the assessment of pathological response. During laparotomy and after peritoneal washings and careful inspection of the abdominal cavity, biopsy of all suspicious lesions was performed, and in the case of no evidence of disease, at least 20 random biopsies were taken. Patients who initially had only an explorative laparotomy underwent a second laparotomy if clinically responsive to chemotherapy, and a second cytoreduction was attempted. Follow-up examinations were performed every 3 months.

p27 Immunohistochemistry and Specificity of Immunostaining. At primary surgery, tumor specimens were dissected and fixed for 24 h in neutral buffered formalin. After fixation, blocks were paraffin embedded for routine histology and immunohistochemical studies. Sections of each case were cut at 5-μm intervals, mounted on glass slides, and dried for 30 min at 55°C. All sections were then dewaxed in xylene, rehydrated through graded alcohol series, washed in tap and then distilled water, and treated with 0.3% hydrogen peroxide for 5 min. The sections were then washed in PBS (pH 7.2) and incubated with normal serum as the blocking reagent. Mouse monoclonal antibodies against p27^{Kip1} (clone DCS-72.F6; Neomarker, Fremont, CA) and PCNA (clone PC10; Sigma Chemical Co., Saint Louis, MO) were applied on the slides at the dilution of 1:150 and 1:1000, respectively. The sections were then incubated with the biotinylated goat antimouse IgG and, after washing in PBS, with avidin-biotin-peroxidase complex for 30 min at room temperature. Diaminobenzidine (Vector, Burlingame, CA) was used as the final chromogen, and hematoxylin was used as a counterstain.

The pattern of staining seen with the p27^{Kip1} monoclonal antibody was confirmed on duplicate slides using a polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The strong positive immunostaining of lymphocytes, in all of the sections examined, represented an internal positive control for preservation of the p27 antigenicity in tissues. For negative control, PBS was substituted for the primary antibody.

p27 Scoring. Specificity of p27^{Kip1} staining was assessed by preabsorption with the peptide used to generate it. All immunoreactive cells were considered positive. Two pathologists (J. P. and G. Z.) separately evaluated p27 staining. Expression was classified as positive (staining in ≥5% of cells) or negative (staining in <5% of cells), as described previously (13). At least 20 high-power fields were chosen randomly, and 2000 cells were counted.

p53 immunohistochemical analysis was performed by using the specific monoclonal antibody for p53 (DO-7; Dako, Carpinteria, CA). Cases were scored as positive (staining in ≥1% of cells) or negative (absence of staining), as reported previously (4). Tumors with high and low proliferative index were divided along the median for PCNA, which was 60% of positive cells.

Statistical Analysis. Fisher's exact test for proportion and the χ^2 test were used to analyze the distribution of p27-positive and -negative cases according to clinicopathological

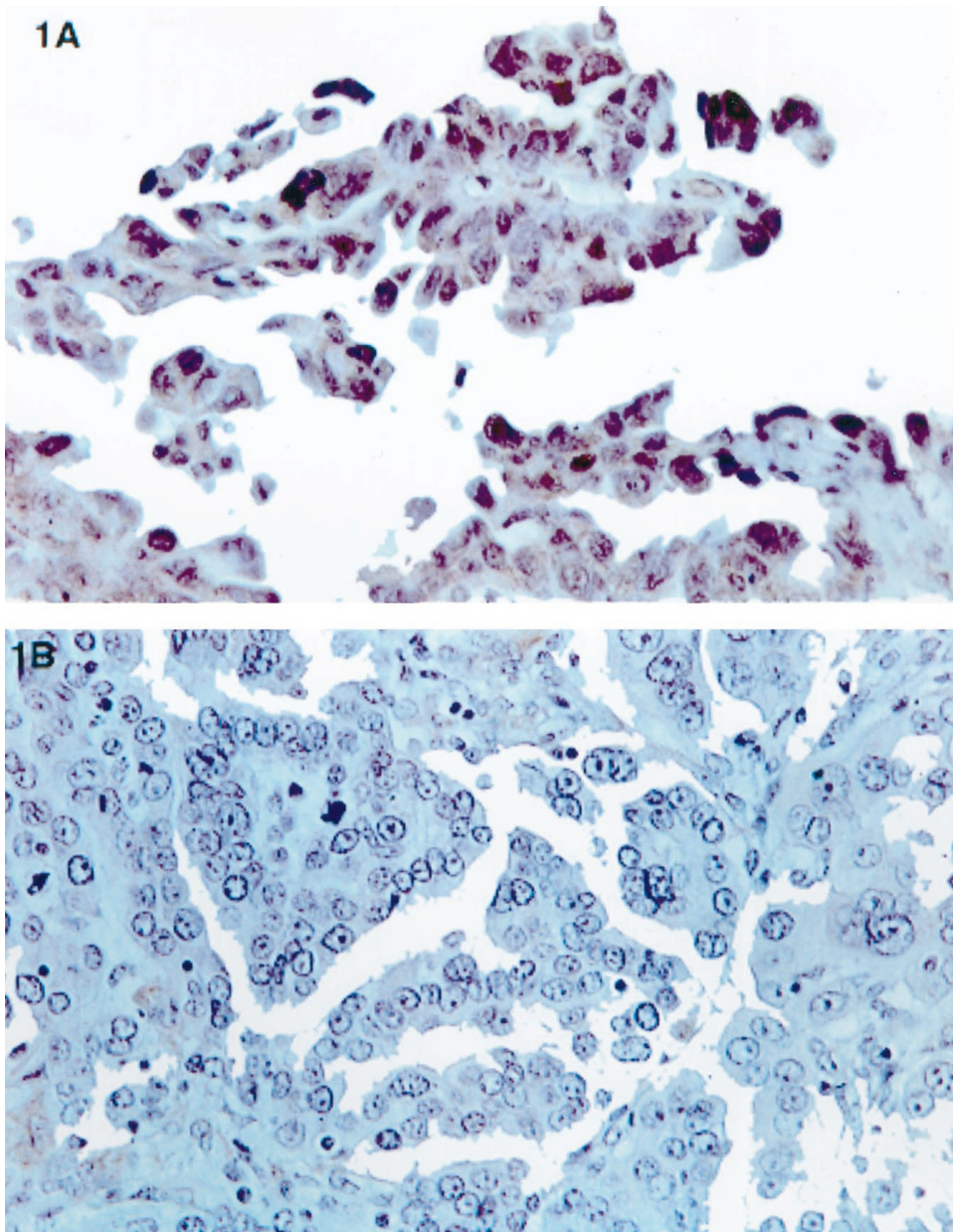


Fig. 1 A, well-differentiated (grade 1) papillary serous carcinoma showing strong nuclear and focal cytoplasmic staining for p27. B, poorly differentiated (grade 3) papillary serous carcinoma negative for p27 ($\times 200$).

characteristics. All medians and life tables were computed using the product-limit estimate, and the curves were examined by the log-rank test (20). Multivariate analysis was performed by the Cox proportional hazards model (21). TTP and OS were calculated from the date of first surgery to the date of clinical or pathological progression or death. Survival analyses were carried out using SOLO Statistical Software (BMDP Statistical Software, Inc., Los Angeles, CA). All reported *Ps* are two sided.

RESULTS

p27 Protein Expression in Epithelial Ovarian Tumors.

A total of 99 primary epithelial ovarian tumors were evaluated. The majority of tumors displayed heterogeneous p27 expres-

sion, whereas only a small group of well-differentiated and poorly differentiated tumors showed present (Fig. 1A) and decreased or absent p27 expression (Fig. 1B), respectively. In contrast to normal epithelium, where p27 is mostly localized in the nuclei, in ovarian adenocarcinomas a concomitant weak cytoplasmic staining was also observed in some cases.

Primary ovarian adenocarcinomas expressed p27 ($\geq 5\%$ positive cells) in 47 of 99 cases (47%), whereas lack of p27 protein expression ($< 5\%$ positive cells) was observed in the remaining 52 cases (53%). The distribution of p27-positive cases according to clinicopathological parameters is shown in Table 1. No differences in p27 expression was found according to age, histology, stage of disease, grade of differentiation,

Table 2 Relationship among the different clinicopathological features in primary advanced ovarian carcinomas

Age (≤ 60 vs. > 60)	–					
Stage (III vs. IV)	0.1	–				
Ascites	NS	0.18	–			
No						
Yes						
Residual tumor	0.13	0.0042	0.0006	–		
≤ 2 cm						
> 2 cm						
Grading	0.12	NS	NS	NS	–	
G1,2 vs. G3						
p27	NS	0.20	NS	0.1	NS	–
Positive						
Negative						
	Age	Stage	Ascites	Residual tumor	Grading	p27

presence of ascites, residual tumor at primary surgery, PCNA, or p53 status. p53 expression did not correlate with any clinicopathological parameter.

Patients who responded to chemotherapy were more frequently (53%) p27 positive than cases who did not respond (27%), although no statistical significance was reached ($P = 0.089$). In the group of optimally cytoreduced patients, cases who achieved complete/partial response to chemotherapy were found to be more frequently p27 positive (62%) than not responding patients (14%; $P = 0.035$).

The reciprocal relationships among the different clinicopathological features is summarized in Table 2. A highly significant association was found only between the presence of residual tumor at first surgery > 2 cm and stage IV disease ($P = 0.004$) and the presence of ascites at diagnosis ($P = 0.0006$).

Survival Analysis. Follow-up data were available for all 99 patients (median follow-up, 27 months; range, 1–84 months). During the follow-up period, progression and death of disease were observed in 58 and 35 patients, respectively. Fig. 2A shows the TTP curve in relation to p27 status. p27-positive cases showed a more favorable prognosis with respect to p27-negative patients; the 5-year TTP rate was 50%, (95% CI, 33–68) for p27-positive cases, compared with 11% (95% CI, 0–22) of p27-negative cases ($P = 0.0004$). Median TTP was 35 months for p27-positive patients as compared with 16 months for p27-negative patients.

Fig. 2B shows the OS curve in relation to p27 status. p27-positive cases showed a 53% (95% CI, 28–78) 5-year OS rate compared with 43% (95% CI, 26–60) of p27-negative cases ($P = 0.0302$). Median OS was not reached in p27-positive cases as compared with 51 months in p27-negative patients.

The prognostic role of age at diagnosis, stage, histological grade, presence of ascites, residual tumor, and p27 status were tested in univariate and multivariate analyses for both TTP (Table 3) and OS (Table 4). Loss of p27 was found to be a strong predictor of a shorter TTP on univariate analysis (RR, 2.63; 95% CI, 1.49–4.47; $P = 0.0007$). Univariate analysis also showed residual tumor ($P = 0.0004$) and stage of disease ($P = 0.01$) to be significantly associated with a short TTP.

In the multivariate analysis of TTP, presence of > 2 cm of residual tumor after surgery and absence of p27 immunoreac-

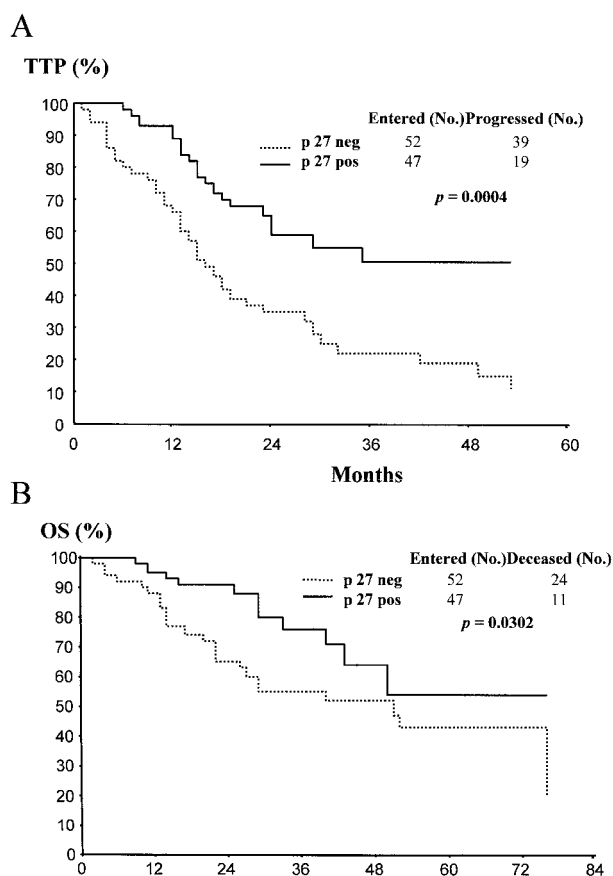


Fig. 2 Kaplan-Meier curve for TTP (A) and OS (B) in 99 primary ovarian carcinomas stratified according to p27 expression. Loss of p27 expression was significantly associated with progression ($P = 0.0004$ by log-rank test) and death of disease ($P = 0.0302$ by log-rank test).

tivity retained independent, negative prognostic roles. In particular, p27-negative cases had a relative risk of progression of disease of 2.61 (95% CI, 1.48–4.61; $P = 0.0009$) with respect to p27-positive tumors. As far as OS is concerned, both residual tumor > 2 cm at first surgery and p27-negative status were demonstrated to be associated with a poor OS in univariate analysis. In multivariate analysis, p27 status was shown to retain

Table 3 Univariate and multivariate analysis of TTP in primary ovarian cancer patients

	Univariate				Multivariate ^b			
	RR1 ^a	95% CI	χ ²	P	RR2	95% CI	χ ²	P
Age (yr)								
≤60	1 ^c							
>60	1.33	0.78–2.28	1.13	.28				
Stage								
III	1 ^c				1 ^c			
IV	2.07	1.12–3.82	5.47	.01	1.21	0.62–2.37	0.33	.50
Grade								
1–2	1 ^c							
3	1.07	0.52–2.20	0.04	.83				
Ascites								
No	1 ^c				1 ^c			
Yes	1.63	0.94–2.84	3.08	.07	1.49	0.82–2.72	1.73	.18
Residual tumor (cm)								
≤2	1 ^c				1 ^c			
>2	2.67	1.55–4.57	12.75	.0004	2.08	1.11–3.89	5.35	.02
p27 status								
Positive	1 ^c				1 ^c			
Negative	2.63	1.49–4.47	11.44	.0007	2.61	1.48–4.61	10.99	.0009

^a RR1, unadjusted RR; RR2, adjusted RR taking into account all the factors listed.

^b Only variables significant in the univariate analysis at a *P* < 0.20 were included in the analysis.

^c Reference category.

Table 4 Univariate and multivariate analysis of OS in primary ovarian cancer patients

	Univariate				Multivariate ^b			
	RR1 ^a	95% CI	χ ²	P	RR2	95% CI	χ ²	P
Age (yr)								
≤60	1 ^c							
>60	1.08	0.52–2.23	0.05	0.81				
Stage								
III	1 ^c				1 ^c			
IV	1.79	0.83–3.86	2.22	0.13	1.08	0.46–2.49	0.03	.85
Grade								
1–2	1 ^c							
3	1.88	0.38–2.03	0.08	0.76				
Ascites								
No	1 ^c				1 ^c			
Yes	1.69	0.80–3.57	1.92	0.16	1.59	0.72–3.49	1.34	.24
Residual tumor (cm)								
≤2	1 ^c				1 ^c			
>2	2.57	1.28–5.16	7.10	0.0077	2.02	0.93–4.41	3.17	.07
p27 status								
Positive	1 ^c				1 ^c			
Negative	2.37	1.12–4.99	5.20	0.0225	2.30	1.07–4.96	4.59	.0032

^a RR1, unadjusted RR; RR2, adjusted RR taking into account all the factors listed.

^b Only variables significant in the univariate analysis at a *P* < 0.20 were included in the analysis.

^c Reference category.

the most significant association with clinical outcome. Patients with p27-negative tumors had a significantly higher RR of dying of disease (2.30; 95% CI, 1.07–4.96; *P* = 0.0032) than patients with p27-positive tumors.

Fig. 3 shows TTP (Fig. 3A) and OS (Fig. 3B) curves after stratification of patients according to p27 status and residual tumor; the presence of p27-positive immunostaining can identify patients with a better clinical outcome in the subgroup of optimally cytoreduced patients as well as in the subgroup of suboptimally cytoreduced patients.

DISCUSSION

We demonstrated that expression of the cdk inhibitor p27 is a strong predictor of longer time to progression and overall survival in a large series of advanced stage ovarian cancer patients. These findings are consistent with most of the studies analyzing the prognostic role of p27 expression in several tumor types. In particular, loss of p27 expression significantly increases the risk of recurrence and death from disease in breast (7), prostate (8), bladder (9), hepatocellular (10), and colorectal

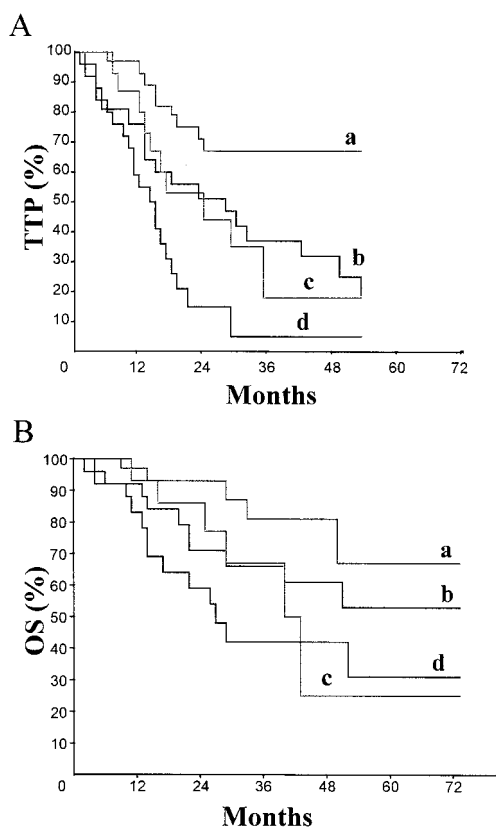


Fig. 3 Probability of progression (A) and survival (B) in 99 patients with stages III–IV ovarian carcinoma as stratified by residual tumor and p27 status. Comparison between the groups by the log-rank test. A: residual tumor (cm) ≤ 2 , p27-positive (a) versus residual tumor ≤ 2 , p27-negative (b): $P = 0.009$; residual tumor (cm) > 2 , p27-positive (c) versus residual tumor > 2 , p27-negative (d): $P = 0.02$; residual tumor (cm) ≤ 2 , p27-negative (b) versus residual tumor > 2 , p27-negative (d): $P = 0.015$. B: residual tumor (cm) ≤ 2 , p27-positive (a) versus residual tumor > 2 , p27-positive (c): $P = 0.047$; residual tumor (cm) ≤ 2 , p27-positive (a) versus residual tumor > 2 , p27-negative (d): $P = 0.0029$. All remaining comparisons are not statistically significant.

(11) carcinomas. Whereas p27 expression appears to be an important predictor of clinical behavior in several malignancies, evidence to date suggests that loss of p27^{Kip1} protein is not attributable to structural alterations of the gene (22) but may result from increased degradation of the protein mediated by the ubiquitin-proteasome pathway (11, 12, 23).

In ovarian cancer, our data extend and confirm preliminary observations by us (13) and others (16) but seem in contrast with a recent study (17), which found lack of independent prognostic significance of p27 in a series of 185 patients with stage III ovarian cancer. The use of a different antibody, the different cutoff value considered for p27 positivity, and the selected stage of patients (stage III only) in that study may account for the different results from our report. However, in the same study, when a complete loss of p27 protein expression was considered versus any degree of p27 expression, a tendency toward a statistically significant association between p27 positivity and a better clinical outcome was found (17).

The negative prognostic role of loss of p27 expression is

not related to its association with unfavorable clinicopathological features nor biological markers of aggressiveness, such as p53 overexpression, as also reported by Newcomb *et al.* (16). Interestingly, there was no correlation (positive or inverse) between p27^{Kip1} and PCNA, suggesting that expression of p27^{Kip1}, as observed previously in other tumors (7, 24), is not merely a reflection of low proliferation rate. Moreover, the association between loss of p27 expression and poor survival remained significant after stratification according to residual tumor at first surgery, a parameter that plays a major role in affecting response to chemotherapy and survival (19). Finally, we demonstrated that loss of p27 expression retained an independent, negative prognostic role also in the multivariate analysis for both TTP and OS. We observed that p27-positive cases showed a higher percentage of response to chemotherapy, especially in the group of patients optimally cytoreduced at first surgery. This finding could justify the apparent closer association between p27 status and TTP with respect to OS, because it is well recognized that TTP more adequately reflects the efficacy of first-line chemotherapy. Platinum-based chemotherapy induces apoptosis in tumor cells, and reduced susceptibility to apoptosis has been proposed as a major mechanism responsible for resistance to chemotherapy (3, 25). Recent evidence indicates that p27 overexpression induces apoptosis in several different human cancer cell lines through a p53-independent pathway (26). Furthermore, in a large series of human breast cancer specimens, p27 levels showed a strong correlation with the apoptotic index and predictive value for the benefits of chemotherapy (27). It is conceivable that p27 expression may confer sensitivity to apoptosis in a p53-independent manner, thus increasing the sensitivity of ovarian cancer cells to chemotherapeutic agents. Further *in vitro* and *in vivo* studies are required to elucidate whether alterations in p27 expression are responsible for the reduced response to chemotherapy in ovarian cancer cells. Moreover, it could be of interest to assess simultaneously other factors, such as the cell cycle control proteins cyclin D1 and cyclin E, which have been postulated to regulate expression levels of p27^{Kip1} in a negative regulatory feedback loop (28).

In summary, in this study, we present evidence for a role of p27 in ovarian cancer patients as an independent prognostic predictor of clinical outcome. Patients with ovarian cancer that show loss of p27 expression are at higher risk of progression and death of disease and may eventually benefit from more aggressive adjuvant therapy. The reliability of p27 as a potential marker in the clinical routine assessment and management of women with advanced ovarian cancer deserves to be further evaluated in long-term follow-up studies.

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