Histol Histopathol (2003) 18: 761-770

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Evaluation of the cyclin-dependent kinase inhibitor $p21^{Cip1}$ in epithelial ovarian tumors of low malignant potential and adenocarcinomas

I. Vassilopoulos, P. Korkolopoulou, A.E. Konstantinidou, E. Patsouris, C. Eftichiadis, I. Thymara, M. Perdiki, K. Pavlakis, E. Agapitos and P.S. Davaris

Department of Pathology, National and Capodistrian University of Athens, Athens, Greece

Summary. Objective: In view of the controversial information on the significance of the cyclin-dependent kinase inhibitor $p21^{Cip1}$ in ovarian cancer, we conducted a retrospective investigation to clarify the relationships of this protein to proliferation rate, clinicopathological variables and prognosis of epithelial ovarian tumors. Methods: Paraffin-embedded tissue from 43 ovarian tumors of low malignant potential (LMP) and 82 primary ovarian adenocarcinomas were stained immunohistochemically for $p21^{Cip1}$, p53 protein and Ki-67 antigen (a marker of cell proliferation). Results: $p21^{Cip1}$ levels were significantly higher in LMP tumors ($p<0.001$) as well as in early stage adenocarcinomas ($p=0.021$) and those associated with minimal residual disease ($p=0.008$). However, no relationship existed between $p21^{Cip1}$ expression and the proliferation rate of adenocarcinomas or LMP tumors. In the vast majority of LMP tumors $p21^{Cip1}$ expression was not accompanied by p53 accumulation. This $p21^{Cip1}$-positive/p53-negative phenotype prevailed in the early stage ($p=0.026$), lower grade ($p=0.018$) adenocarcinomas as well as in those left with minimal residual disease ($p=0.059$). In patients with lower grade adenocarcinomas, decreased $p21^{Cip1}$ expression was adversely related to poor overall survival on its own ($p=0.0500$) and when combined with p53 protein overexpression ($p=0.0323$). In multivariate analysis, only the stage remained as the independent predictor of survival. Conclusions: Decreased $p21^{Cip1}$ expression is related to several indicators of aggressiveness in ovarian adenocarcinomas and seems to be differentially regulated in LMP tumors and adenocarcinomas. On the contrary, deregulation of $p21^{Cip1}$ expression does not seem to participate in the pathogenesis of LMP tumors. Furthermore, although $p21^{Cip1}$ alone or combined with p53 is of prognostic significance in lower grade adenocarcinomas, it does not appear to add to the information gained from traditional prognosticators.

Key words: $p21^{Cip1}$, Immunohistochemistry, Ovarian carcinomas, LMP, Prognosis

Offprint requests to: Penelope Korkolopoulou, 73, Vassileos Pavlou Str., GR-154 52 Psychico, Athens, Greece. Fax: +302107462157. e-mail: pkorkol@cc.uoa.gr

Introduction

Ovarian cancer is the most common cancer in women to be diagnosed at advanced stage and is the leading cause of death from gynecological malignancy in western countries (Landis et al., 1999). Despite optimal surgical debulking and a pathologically complete response to primary chemotherapy, half of the patients will develop a recurrence and will eventually die of their disease within 2 years, denoting the inadequacy of traditional prognosticators in predicting clinical outcome in individual cases (Landis et al., 1999). Therefore, there has been a growing interest in the characterization of more reliable prognostic factors closely related to tumor cell biology, which would assist in the identification of patients who could benefit from more aggressive therapy.

Although our understanding of the molecular mechanisms intervening and conducting the biological behavior of ovarian carcinomas is certainly incomplete, the intuitive notion that ovarian cancer, like most cancers, arises as a result of perturbations in cell-cycle regulation has rapidly been appreciated (Weinberg, 1991). In particular, disruption of the mechanisms regulating transition from G1 to S phase appears to be a key issue in cancer evolution. Gaining insight into the molecular machinery coordinating orderly progression into S phase has unraveled the crucial role of various inhibitory proteins, known as cyclin-dependent kinase inhibitors (CKIs), which bind to and down-regulate the activities of cyclin/cyclin-dependent kinase (Cdk) complexes required for driving the cell through the various restriction points of the cell cycle (Granna and Reddy, 1995; Sherr, 1996). Currently, two groups of CKIs have been identified: the Cip/Kip family, with

broad cyclin-Cdk reactivity, and the INK4 family with a much more selective inhibitory effect on cyclin D-Cdk 4/6 complexes.

The WAF1/Cip1/SD1 gene product p21 is a member of the Cip/Kip family of CKIs, able to arrest the cell cycle at the G1 checkpoint, by interacting with cyclin/cdk complexes as well as with the DNA polymerase δ replicating factor proliferating cell nuclear antigen (PCNA) (Zhang et al., 1994; Luo et al., 1995). The inhibitory function of $p21^{Cip1}$ on DNA synthesis requires the formation of quarternary complexes composed of cyclins, CDKs and PCNA (Zhang et al., 1993) and is thought to be stoichiometrically regulated, being exerted only when $p21^{Cip1}$ is in molar excess. If the ratio of $p21^{Cip1}$ to cdk is less than one, $p21^{Cip1}$ serves only as an assembling factor for cdk complex and does not inhibit cdk activity. $p21^{Cip1}$ binding prevents CDK2/cyclin E-catalysed phosphorylation of retinoblastoma gene protein (pRb) with consequent abrogation of pRb function as a negative regulator of G1/S restriction point (Cordon-Cardo, 1995). Evidence has been provided that the WAF1 gene possesses at least two p53-responsive sites in its promoter region. Binding of p53 to these elements activates the transcription of $p21^{Cip1}$, suggesting that the latter is a potent downstream mediator of the antiproliferative function of the wild-type p53 (El-Deiry et al., 1993). On the other hand, $p21^{Cip1}$ induction also occurs in a p53-independent manner, having been observed in various normal tissues during development or differentiation in the absence of p53 activation and in tumor cells with mutated p53 (Johnson et al., 1994; Macleod et al.,1995; Parker et al., 1995).

Recently, a number of investigators have looked into $p21^{Cip1}$ expression in ovarian cancer with contradictory findings (Anttila et al., 1999; Costa et al., 1999; Shimizu et al., 1999; Werness et al., 1999; Ferrandina et al., 2000 Schmider et al., 2000; Sengupta et al., 2000). This controversial information regarding the clinical significance of $p21^{Cip1}$ in ovarian carcinomas has prompted us to undertake the present study. The aim was to determine the possible associations of $p21^{Cip1}$ expression with clinicopathological parameters, p53 expression and patients' outcome in a large series of epithelial ovarian tumors.

Materials and methods

Patients

A total of 125 consecutive cases of primary epithelial ovarian tumors of low malignant potential (LMP) and adenocarcinomas, diagnosed and treated at Alexandra General Hospital and Iasso Gynecological Institute of Athens between 1989 and 1999, were enrolled in the present study. Eighty-two specimens were defined as ovarian adenocarcinomas, whereas 43 specimens fulfilled the criteria of LMP tumors (Scully and Sobin, 1987; Russel, 1994). Histological classification of tumors was carried out according to the World Health Organization System (Scully and Sobin, 1987). Poorly differentiated adenocarcinoma was the diagnosis for those adenocarcinomas that did not show evident cellular differentiation. Adenocarcinomas were graded as well, moderately and poorly differentiated (Scully and Sobin, 1987) and patients were assigned a clinical stage according to the International Federation of Gynecology and Obstetrics (FIGO, 1989) standards. Surgical and pathological findings and postoperative abdomino-pelvic computerized tomography (CT) scans were used to determine the FIGO stage for the ovarian adenocarcinomas and the residual disease after the initial surgery. Persistence of tumor masses of <2 cm was defined as minimal residual disease, whereas the presence of masses of >2 cm in diameter was defined as bulk residual disease (Anttila et al., 1999; Sengupta et al., 2000).

All patients with carcinomas had undergone total abdominal hysterectomy with bilateral salpingo-oophorectomy followed by chemotherapy. None had received preoperative chemotherapy or radiothrerapy. The clinicopathological characteristics of these patients are summarized in Table 1. Follow-up information was available in 71 patients. By the time this study was undertaken, 22 patients had died of their disease after a median survival of 24 (1-60) months whereas the median follow-up period for the remaining 49 patients was 52.5

Table 1. Clinical data of patients with ovarian adenocarcinomas.

Age (years)	
Median (range)	53 (20-78)
Histological type	
Serous	29 (35%)
Mucinous	10 (12%)
Endometrioid	17 (21%)
Clear cell	19 (23%)
Adenocarcinoma poorly differentiated	7 (9%)
Histological grade	
1	15 (18%)
2	39 (48%
3	28 (34%)
FIGO stage	
I	31 (38%)
II	4 (5%)
III	36 (44%)
IV	7 (9%)
Not specified	4 (5%)
Primary residual tumor	
None	35 (43%)
<2 cm	10 (12%)
>2 cm	37 (45%)
Adjuvant chemotherapy	
Platinum-containing	57 (70%)
Non platinum-containing	21 (26%)
No data	4 (5%)
Clinical outcome	
Died of ovarian cancer	22 (27%)
Alive	49 (60%)
Lost to follow-up	11 (13%)

(14-126) months.

Immunohistochemistry

Tissues were fixed immediately after removal in 10% formalin and embedded in paraffin. Sections for immunohistochemistry were cut at 3 μm, mounted on Superfrost/Plus glass slides and left to dry overnight at 37 °C. All cases were stained for $p21^{Cip1}$, p53 and Ki-67 using the following monoclonal antibodies: 4D10 for $p21^{Cip1}$ (Novocastra, Burlingame, CA) diluted 1:20; D0-1 for p53 (Dako, Carpinteria, CA) diluted 1:100; and MIB-1 for Ki-67 (YLEM s.r.l., Rome, Italy) purchased prediluted by the manufacturer. The incubation time was 1 hour at room temperature for p53 and Ki-67 and 18 hours at 4 °C for $p21^{Cip1}$. Before applying the primary antibody, sections were treated in a microwave oven at 750 W for a total of 30 minutes (three cycles of 10 minutes each) in 10 mmol/L sodium citrate buffer pH 6.0. Slides were left to cool down in the antigen retrieval solution for 20 minutes. Then, the standard streptavidin-biotin complex immunoperoxidase technique (Histostain-SP Kit, Zymed Laboratories, San Francisco, CA) was used. Peroxidase activity was visualized with chromogen 3,3'-diaminobenzidine tetrachloride in 0.5 mmol/L Tris Buffer. The slides were counterstained with hematoxylin. Known positive controls (a colorectal carcinoma for DO-1, normal tonsillar tissue for MIB-1 and a previously irradiated piece of skin for 4D10) as well as negative controls (sections in which the primary antibody was substituted with non-immune mouse serum) were also stained in each run.

Assessment of staining

Staining for all antibodies was assessed by two observers without any knowledge of the clinical data. Whenever a difference of greater than 5% was observed between the two readings, slides were reviewed jointly and a consensus was reached. To this end, nuclei from about 2000 tumor cells from 20 systematically randomized fields (x400) throughout the whole section were counted and the labeling index (LI) was calculated as the percentage of labeled nuclei out of the total number of tumor cells counted. All clearly identifiable nuclear staining beyong background was recorded as positive for Ki-67 (Anttila et al., 1999; Palazzo et al., 2000), but for p53 and $p21^{Cip1}$ the threshold of positivity was raised to 10% and 1% respectively, according to published convention (Palazzo et al., 2000; Schmider et al., 2000; Sengupta et al., 2000). Given that the immunostaining intensity did not significantly vary from case to case, the expression of various markers was assessed quantitatively.

Statistical analysis

$p21^{Cip1}$ LI, Ki-67 LI and p53 LI were considered as continuous variables. The relationships between the expression of the three markrers and clinicopathological parameters were examined by the Mann-Whitney U-test and Kruskal-Wallis analysis of variance. The associations of the categorized form of $p21^{Cip1}$ (<1% vs ≥1%) and p53 (<10% vs ≥10%) were tested with Pearson's chi-square test with continuity correction and Fisher's exact test. The interrelations among p53, $p21^{Cip1}$ and Ki-67 levels were analysed with Spearman's correlation coefficient.

Survival analysis was restricted to adenocarcinomas, given that LMP tumors follow a substantially more favorable clinical course. The prognostic effect of the various parameters (age, histological type, grade, FIGO stage, volume of residual disease, type of chemotherapy, Ki-67 LI, p53 expression, $p21^{Cip1}$ LI and the combined $p21^{Cip1}$/p53 phenotype) was tested by univariate and multivariate analysis. Univariate analysis was based on life tables and differences among groups were tested for significance using the log-rank test. Age was categorized on the basis of the median value. p53 and $p21^{Cip1}$ expression was categorized as positive versus negative using 10% and 1% as cut-off points respectively. Ki-67 LI was categorized on the basis of 10% (Palazzo et al., 2000; Sengupta et al., 2000). Multivariate analysis was performed using the stepwise Cox's regression model (forward selection of variables) to evaluate the predictive power of each variable independently of the others. In order to avoid any "data-driven" categorization, numerical variables were entered in continuous form only. Statistical calculations were performed using the SPSS for Windows Software (SPSS Inc., Chicago IL). A p-value of less than or equal to 0.05 was considered indicative of a statistically significant difference.

Results

$p21^{Cip1}$ expression in LMP tumors and ovarian adenocarcinomas

Using 1% as the cut-off point, positive staining for $p21^{Cip1}$ was observed in 35/43 (81.4%) LMP cases and in 39/82 (47.6%) adenocarcinoma cases (χ^2, p=0.001). $p21^{Cip1}$ LIs ranged from 1% to 90% in the former group and from 1% to 60% in the latter. Positivity was confined to the nucleus and had a granular quality (Fig. 1) The distribution of positive nuclei was uniform throughout the neoplastic tissue. In a few (10%) adenocarcinoma cases a faint cytoplasmic reactivity was also noted which was disregarded as nonspecific. Exclusively cytoplasmic localization of the protein was not observed in our series. Non-neoplastic elements such as ovarian surface epithelium, ovarian cortical cells, lymphocytes and endothelial cells were not labeled. The median $p21^{Cip1}$ LI was significantly higher in LMP tumors (8%) compared to carcinomas (3%) (Mann-Whitney U-test, p<0.001, Table 2). When comparisons between LMP cases and carcinomas were made for

serous and mucinous types separately, a difference in the incidence of $p21^{Cip1}$ positivity between carcinomas and LMP tumors was recorded for serous (Fisher's exact test, p<0.001; Mann-Whitney U-test, p<0.001), but not for mucinous type (Fisher's exact test, p=0.240; Mann-Whitney U-test, p=0.248) (Table 2). However, mucinous LMP tumors were less often (57.1%) $p21^{Cip1}$-positive than serous ones (93.1%) (Fisher's exact test, p=0.009).

Correlation of $p21^{Cip1}$ expression in adenocarcinomas with clinicopathological parameters

The rate of $p21^{Cip1}$ positivity significantly decreased with advancing stage (I-II versus III versus IV) (χ^2, p=0.044) as well as with bulk residual disease (χ^2, p=0.023) (Table 3) but was not related to tumor grade (χ^2, p=0.423). The same applied to $p21^{Cip1}$ LI (Mann-Whitney U-test, p=0.021, p=0.008, p=0.294 respectively). There was an almost significant relationship between $p21^{Cip1}$ expression and histological type (χ^2, p=0.059), in the sense that serous and mucinous types tended to be less often positive than clear-cell and endometrioid types. $p21^{Cip1}$ levels were also lower in the former two types (Kruskal-Wallis ANOVA, p=0.042)

p53 immunostaining

p53 immunoreactivity was confined to tumor cell nuclei. Normal surface epithelium as well as ovarian cortical cells and inflammatory cells did not express p53. According to the 10% cut-off point, 39 out of 82 (47.6%) adenocarcinomas and only one out of 43 (2.3%) LMP tumors were considered p53-positive (x^2, p<0.001). Serous adenocarcinomas were more often p53-positive than their LMP counterparts (χ^2, p<0.001), but mucinous adenocarcinomas were not (χ^2, p=0.355, Table 2). Within the adenocarcinoma group, the rate of p53-positivity significantly increased with high grade (χ^2, p=0.001) and marginally with advancing stage category (χ^2, p=0.075), but remained unaffected through histological types (χ^2, p=0.250) and residual disease (χ^2,

Table 2. $p21^{Cip1}$, p53 and Ki-67 expression in LMP tumors and ovarian adenocarcinomas.

HISTOLOGY	$p21^{Cip1}$ EXPRESSION		p53 EXPRESSION		Ki-67 LI (%)
	Pos/neg	$p21^{Cip1}$LI (%) (median,range)	Pos/neg	p53 LI (%) (median, range)	(median, range)
LMP tumors (n=43)	35/8	8.0, 1.0-90.0	1/42	40.0, -	5.0, 0.5-50.1
Serous (n=29)	27/2	8.0, 1.0-90.0	0/29	-, -	3.0, 0.5-12.1
Mucinous (n=14)	8/6	22.5, 1.0-90.0	1/13	40.0, -	10.0, 1.1-50.2
Carcinomas (n=82)	39/43	3.0, 1.0-60.0	39/43	65.0, 10.0-99.2	15.0, 0.8-95.1
Serous (n=29)	9/20	2.0, 1.0-5.0	18/11	67.5, 25.0-99.1	20.0, 0.8-55.1
Mucinous (n=10)	3/7	10.0, 5.0-10.2	3/7	30.0, 20.1-50.2	11.0, 4.0-60.2

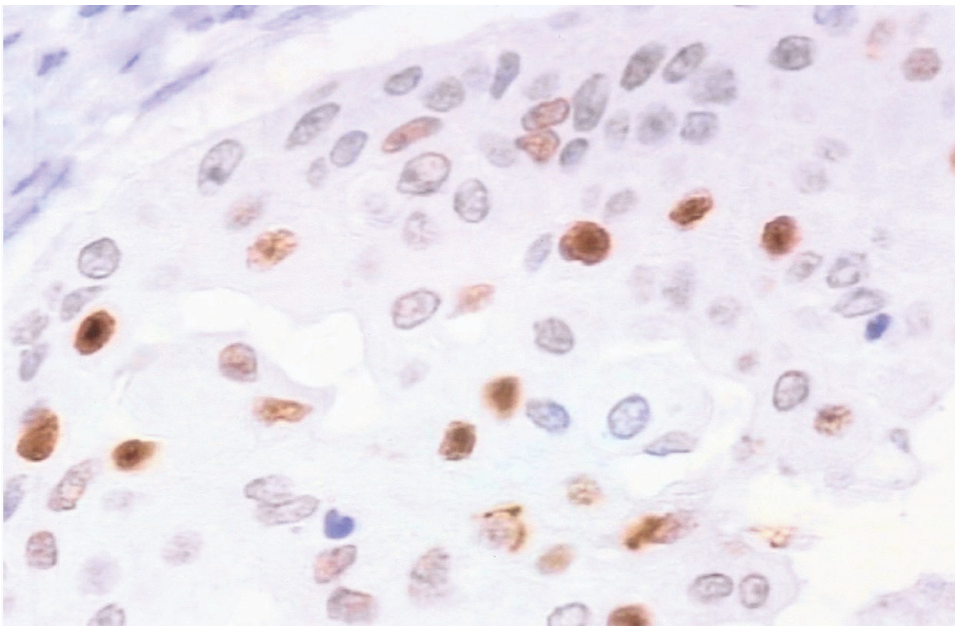

Fig. 1. $p21^{Cip1}$ immunostaining in an endometrioid grade 2 adenocarcinoma. Many neoplastic nuclei are stained. $p21^{Cip1}$LI in this case was 25.5%.x 250

p=0.254). There was also no relationship between p53 LI and histological type (p=0.263), histological grade (p=0.229), FIGO stage (p=0.984) or the volume of residual disease (p=0.572) (Table 3).

Ki-67 immunostaining

Ki-67 immunoreractive cells were readily identified in all cases but Ki-67 LI was much higher in adenocarcinomas compared with LMP tumors (Mann-Whitney U-test, p<0.001) and in mucinous LMP compared to serous LMP tumors (p=0.001) (Table 2). High grade adenocarcinomas displayed higher percentages of Ki-67-positive cells (Kruskal- Wallis test, p=0.002) but Ki-67 LI was unrelated to stage (p=0.082) or histological type (p=0.598). In addition, higher proliferation rate correlated with bulk residual disease (Mann-Whitney U-test, p=0.002) (Table 3).

Table 3. $p21^{Cip1}$, p53 and Ki-67 expression as related to clinicopathological variables in ovarian adenocarcinomas.

VARIABLE	$p21^{Cip1}$ EXPRESSION		p53 EXPRESSION		Ki-67 LI (%)
	Pos/neg	$p21^{Cip1}$LI (%) (median,range)	Pos/neg	p53 LI (%) (median, range)	(median, range)
Histological type					
Serous (n=29)	9/20	2.0, 1.0-5.0	18/11	67.5, 25.0-99.1	20.0, 0.8-55.1
Mucinous (n=10)	3/7	10.0, 5.0-10.0	3/7	30.0, 20.1-50.2	11.0, 4.0-60.2
Endometrioid (n=17)	12/5	2.5, 1.0-35.0	8/9	65.0, 10.1-95.2	15.0, 8.2-80.3
Clear cell (n=19)	11/8	4.0, 1.0-60.0	6/13	45.0, 10.0-90.1	12.0, 4.1-60.3
Adenocarcinoma poorly differentiated (n=7)	4/3	2.5, 2.0-20.0	4/3	67.5, 60.0-90.3	20.0, 8.2-95.1
Histological grade					
1 (n=15)	9/6	3.0, 1.0-10.0	2/13	52.5, 30.2-75.1	12.0, 0.8-70.1
2 (n=39)	19/20	5.0, 1.0-60.0	17/22	50.0, 10.0-99.2	12.0, 3.1-65.2
3 (n=28)	11/17	2.0, 2.0-20.0	20/8	70.0, 10.0-99.1	37.5, 5.1-95.2
FIGO stage					
I/II (n=35)	22/13	3.0, 1.0-60.0	12/23	60.0, 10.0-99.2	12.0, 0.8-70.3
III (n=36)	12/24	2.5, 1.0-20.0	22/14	62.5, 10.0-99.0	20.0, 3.0-95.2
IV (n=7)	3/4	2.0, 1.0-10.0	3/4	50.0, 30.1-95.2	35.0, 3.0-75.1
Primary residual tumor					
Absent or minimal (n=45)	27/18	3.0, 1.0-60.0	18/27	70.0, 10.0-99.1	12.0, 0.8-70.1
Bulk (n=37)	12/25	3.0, 1.0-20.0	21/17	60.0, 10.0-95.2	30.0, 3.0-95.2

Table 4. Associations of the combined $p21^{Cip1}$/p53 expression with clinicopathological variables of ovarian tumors.

VARIABLE	$p21^{Cip1}$-/p53+	$p21^{Cip1}$-/p53-	$p21^{Cip1}$+/p53-	$p21^{Cip1}$+/p53+
Histology				
LMP tumors	2	6	35	0
Adenocarcinomas	24	19	24	15
	p<0.001 (df=3)			
Histological type				
Serous	15	5	6	3
Mucinous	1	6	1	2
Endometrioid	2	3	6	6
Clear cell	4	4	9	2
Adenocarcinoma poorly differentiated	2	1	2	2
	p=0.018 (df=12)			
Histological grade				
1/2	11	15	20	8
3	13	4	4	7
	p=0.018 (df=3)			
FIGO stage				
I/II	5	8	15	7
III/IV	17	11	7	8
	p=0.026 (df=3)			
Primary residual tumor				
Absent or minimal	9	9	18	9
Bulk	15	10	6	6
	p=0.059 (df=3)			

Correlations among p21Cip1, p53 and proliferation rate

A moderately strong negative correlation emerged between $p21^{Cip1}$ expression and proliferation rate (rho=-0.337, p<0.001) as well as between $p21^{Cip1}$ and p53 (rho=-0.347, p<0.001). When, however, LMP tumors and adenocarcinomas were examined separately, the relationship between $p21^{Cip1}$ and proliferation rate disappeared. p53 expression, on the other hand, exerted a positive, though less strong, effect on proliferation rate (rho=0.496, p<0.001).

Combined p21Cip1 /p53 expression

According to the combined $p21^{Cip1}$/p53 expression, our cases were divided into four subsets: $p21^{Cip1}$-positive/p53-negative, $p21^{Cip1}$-negative/p53-positive, $p21^{Cip1}$-positive/p53-positive and $p21^{Cip1}$-negative/p53-negative. The vast majority (81.4%) of LMP tumors belonged to the $p21^{Cip1}$-positive/p53-negative group whereas in adenocarcinomas $p21^{Cip1}$-positive/p53-negative and $p21^{Cip1}$-negative/p53-positive phenotypes were equally common (28%) (χ^2, p<0.001) (Table 4). The distribution of these subsets was also significantly different across the various grade categories (χ^2, p=0.018), in the sense that the $p21^{Cip1}$-positive/p53-negative phenotype prevailed in grades 1 and 2, whereas the $p21^{Cip1}$-negative/p53-positive phenotype was most often encountered in grade 3 cases. The combined $p21^{Cip1}$/p53 phenotype was also related to stage (χ^2, p=0.026) and marginally to the presence of residual tumor (χ^2, p=0.059), with the majority of $p21^{Cip1}$-positive/p53-negative cases presenting with early (I-II) stages and being left with minimal residual tumor after surgery. Most clear-cell adenocarcinomas expressed the $p21^{Cip1}$-positive/p53-negative phenotype in contrast to serous adenocarcinomas, most of which belonged to the $p21^{Cip1}$-negative/p53-positive group (χ^2, p=0.018) (Table 4).

Survival analysis

In univariate survival analysis, high grade (p=0.0387), advanced stage (p=0.0003), the presence of bulk residual tumor (p=0.0010) and increased (>10%) Ki-67 LI (p=0.0127) portended a decreased overall survival in patients with adenocarcinomas (Table 5). $p21^{Cip1}$ failed to reach significance in this regard. However, when patients were stratified by grade, the absence of $p21^{Cip1}$expression exerted an unfavorable effect on the survival of patients with grades 1 and 2 (p=0.0500). The mean survival rate was 66 months for the $p21^{Cip1}$-negative group as opposed to 112 months for the $p21^{Cip1}$-positive one (Fig. 2) The combined $p21^{Cip1}$/p53 expression also affected survival in patients with lower grade adenocarcinomas only (p=0.0323), with $p21^{Cip1}$-negative/p53-positive cases displaying the shorter mean survival time (43 months) among the four $p21^{Cip1}$/p53 phenotypes (Fig 3) All the examined parameters were entered into Cox's analysis. However, stage emerged as the single independent predictor of overall survival (Table 5).

Discussion

The molecular basis of ovarian cancer has been a subject of intense scrutiny in recent years. However, histological and biological heterogeneity, as well as

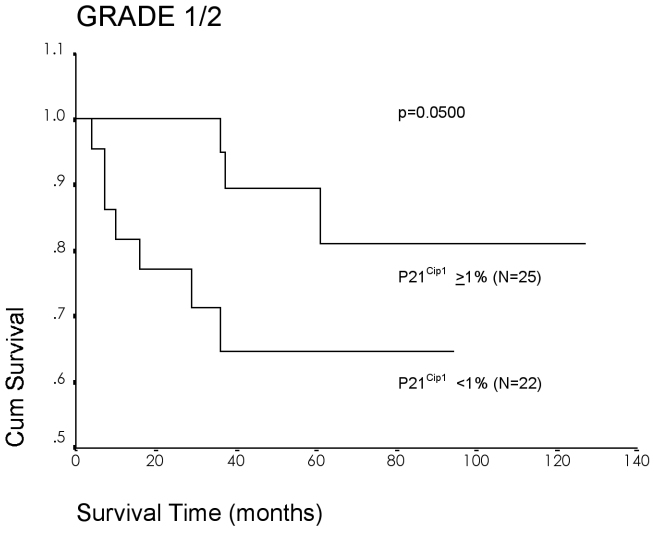

Fig. 2. Overall survival of patients with grade 1 and 2 adenocarcinomas in relation to $p21^{Cip1}$ status. Patients with $p21^{Cip1}$-negativity tended to fare substantially worse than those with $p21^{Cip1}$-positive tumors. (Kaplan Meier curves, p=0.0500).

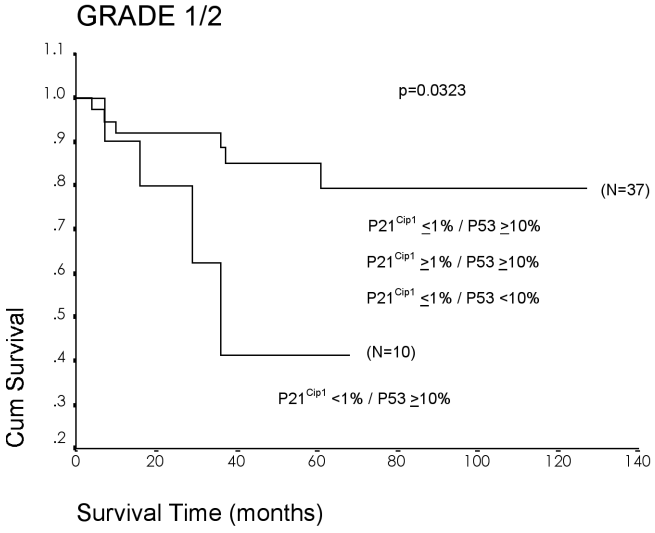

Fig. 3. Overall survival of patients with grade 1 and 2 adenocarcinomas in relation to the dichotomized $p21^{Cip1}$/p53 phenotype. Survival rates were lower in the $p21^{Cip1}$-negative/p53-positive group than in the remaining three phenotypes. (Kaplan Meier curves, p=0.0323).

uncertainties about the cell of origin, have impeded progress in our understanding of the underlying molecular mechanisms in these neoplasms. In search of the key events intervening in the development and progression of ovarian carcinomas, considerable attention has been directed toward the aberrations of cell-cycle regulatory proteins.

In the present study, we selected an immunohistochemical approach to evaluate the expression of $p21^{Cip1}$ in ovarian cancer. Given that mutations in the coding region of WAF1 gene are extremely rare in a wide variety of human tumors (Shiohara et al., 1994), including those of the ovary (Wan et al., 1996), immunohistochemistry provides valuable information on the WAF1 gene functional status. The frequency of $p21^{Cip1}$ expression in ovarian carcinomas (analysed by immunohistochemical and molecular methods) reported in the literature fluctuates between 42% to 78% (Barboule et al., 1995; Anttila et al., 1999; Costa et al., 1999; Werness et al., 1999; Palazzo et al., 2000; Schmider et al., 2000; Sengupta et al., 2000), although in two studies this percentage is much lower (Lukas et al., 1997; Shimizu et al., 1999). In our series the rate of $p21^{Cip1}$ immunopositivity rises to 47.6%, comparing well with most published studies. This finding, in association with the fact that non-neoplastic ovarian tissue, including celomic epithelium, revealed no $p21^{Cip1}$ expression (Werness et al., 1999; Ferrandina et al., 2000), has led some investigators to hypothesize that $p21^{Cip1}$ activation may be associated with ovarian tumorigenesis (Ferrandina et al., 2000), in alignment with data reported by other investigators on lung, bladder, breast and brain tumors (Jung et al., 1995; Korkolopoulou et al., 1998, 2000; Rey et al., 1998; Takeshima et al., 1998).

In LMP tumors, $p21^{Cip1}$ expression was present in higher levels than in adenocarcinomas providing evidence for a different pathogenetic basis of these two major categories of epithelial ovarian tumors. Analogous findings have been recently published by us and other authors (Masciullo et al., 1999; Korkolopoulou et al., 2002) regarding the differential expression of $p27^{Kip1}$, a CKI structurally related to $p21^{Cip1}$, in LMP and adenocarcinomas. This picture is in harmony with the well-known growth inhibitory role of $p21^{Cip1}$ suggested by in vitro data (El-Deiry et al., 1993) and, seemingly, in contrast with the proposed role of $p21^{Cip1}$ in ovarian tumorigenesis as mentioned above (Ferrandina et al., 2000). To explain this disparity between theory and practice, it has been postulated that tumor cells may modify $p21^{Cip1}$ expression attenuating its inhibitory activity (Jung et al., 1995). Alternatively, Ferrandina et al. (2000) have proposed that elevation of $p21^{Cip1}$ might represent a biochemical adaptation aiming to overcome the rapid proliferation rate of tumorous tissue. Additional experiments (i.e. gene transfection, transgenic and knock out experiments) are required to elucidate the role of $p21^{Cip1}$ in ovarian cancer initiation.

Opinions differ as to the association between $p21^{Cip1}$ expression and clinicopathological indicators of aggressiveness in ovarian adenocarcinomas. In most studies, an inverse relationship between $p21^{Cip1}$ levels and stage, grade or volume of residual disease (Anttila et al., 1999; Costa et al., 1999; Ferrandina et al., 2000; Schmider et al., 2000) has been established, but other authors (Werness et al., 1999; Sengupta et al., 2000) have disputed such a relationship. This variation largely results from the different positivity criteria imposed in these studies. In the present investigation, in order to circumvent the danger of using cut-off points, $p21^{Cip1}$ exression was considered a continuous variable and all the associations between loss of $p21^{Cip1}$ and clinicopathological parameters of aggressiveness were also obtained when $p21^{Cip1}$ was tested as a continuous variable. Our findings are consonant with those of the former group of investigations and seem to advocate that $p21^{Cip1}$ loss of function might be a late event in the progression of these tumors. Within LMP tumors, $p21^{Cip1}$ positivity was more often associated with serous histology, strengthening the observations of Palazzo et al. (2000). However, within adenocarcinomas, $p21^{Cip1}$ overexpression prevailed in clear cell and endometrioid categories, in agreement with the data reported by Werness et al. (1997), Anttila et al. (1999), Costa et al.

Table 5. Univariate and multivariate analysis of overall survival.

VARIABLE	UNIVARIATE ANALYSIS (log-rank test)	MULTIVARIATE ANALYSIS (Cox's model)		
		p-value	Hazard ratio (HR)	95% confidence limits
Age (<53/>53)	0.0527	0.134		
Histological type (serous/mucinous/endometrioid/clear cell/adenocarinoma)	0.4488	0.159		
Histological grade (1/2/3)	0.0387	0.136		
FIGO stage (I-II/III/IV)	0.0003	0.002	3.423	1.599-7.327
Residual tumor (absent-minimal/bulk)	0.0010	0.494		
Chemotherapy	0.9951	0.127		
$p21^{Cip1}$ LI (<1%/≥ 1%)	0.2751	0.332		
p53 LI (<10%/≥ 10%)	0.1674	0.580		
$p21^{Cip1}$/p53 ($p21^{Cip1}$+/p53- vs $p21^{Cip1}$+/p53 + or $p21^{Cip1}$-/p53- vs $p21^{Cip1}$-/p53+)	0.3050	0.128		
Ki-67 LI (<10%/>10%)	0.0127	0.239		

(1999) and Shimizu et al. (1999). What emerges from these findings is that the role of $p21^{Cip1}$ in the progression of ovarian tumors may be histology-dependent.

Consistent with the wild p53-dependent activation of $p21^{Cip1}$ (El-Deiry et al., 1993), we observed a significant negative correlation between p53 and $p21^{Cip1}$ expression, lending support to the results by Anttila et al. (1999), Costa et al. (1999) and Sengupta et al. (2000). However, the correlation was not very strong, implying that in a minority of ovarian tumors, $p21^{Cip1}$ is p53-independently regulated. Relevant to this issue are the differences in the distribution of the various $p21^{Cip1}$/p53 phenotypes between LMP and adenocarcinomas as well as among the various grade, stage and residual disease categories. Thus, the $p21^{Cip1}$-positive/p53-negative phenotype characterized the vast majority of LMP tumors as well as adenocarcinomas belonging to early stage, low grade and minimal residual disease categories. The opposite applied to the double-positive $p21^{Cip1}$/p53 phenotype, this being commoner in adenocarcinomas compared to LMP and to grade 3 cases. Given that p53 immunoreactivity in ovarian adenocarcinomas is a surrogate marker for p53 gene mutation (Skilling et al., 1996), and taking into account the limitations due to the lack of p53 gene analysis in our study, we may presume that the concomitant overexpression of p53 and $p21^{Cip1}$ speaks in favor of p53-independent induction of $p21^{Cip1}$, while the expression of $p21^{Cip1}$ in the absence of p53 might be indicative of its p53 dependence (Anttila et al., 1999). Following this line of argument, it sounds logical to hypothesize that the p53-dependent mechanisms of p21 induction operate mainly in LMP tumors and adenocarcinomas of a favorable phenotype, in contrast to the p53-independent mechanisms which, not unexpectedly, are associated with features of aggressive disease.

We also observed a significant negative correlation between $p21^{Cip1}$ expression and proliferation rate in the entire cohort, which, however, disappeared when our cases were stratified into LMP and carcinoma categories. This fact implies that this correlation actually reflected the differences in both proliferation rate and $p21^{Cip1}$ expression between these two categories of tumors. Palazzo et al. (2000) claim that such a lack of correlation between $p21^{Cip1}$ expression and proliferation rate is to be expected in tumors with a low proliferation index, indicating that most cells are still able to exit from the cell-cycle into G0. Interestingly, we obtained similar findings when studying the effect of $p27^{Kip1}$ on the proliferation rate of LMP tumors (Korkolopoulou et al., 2002). Explaining the abrogation of growth-regulatory effect of $p21^{Cip1}$ in tumors with a high content of cycling cells, such as ovarian carcinomas, is more difficult. Probably, in these cases cells are allowed to bypass the G1/S restriction point despite the presence of $p21^{Cip1}$, because of the inability of the latter to form active complexes with cyclin/cdks when its stoichiometry is altered (Barboule et al., 1995). Alternatively, it could be hypothesized that the antiproliferative action of $p21^{Cip1}$ is neutralized by the positive effect exerted by p53 on proliferation rate.

Admittedly, the rather small number of deaths from disease-related causes is a drawback of the present series. However, the proportion of advanced to early stages compares well with previous consecutive series (Anttila et al., 1999; Shimizu et al., 2000) and the results of survival analysis recapitulate many of the factors that have been advanced as important determinants of clinical outcome in ovarian cancer, namely FIGO stage, the volume of residual disease and, to a lesser extent, histological type and grade (Hakes, 1993) which supports that our cohort is representative and that statistical analysis is valid. The prognostic utility of $p21^{Cip1}$ in ovarian carcinomas is a contentious issue and no consensus has been reached thus far. Anttila et al. (1999), Costa et al. (1999), Ferrandina et al. (2000) and Schmider et al. (2000) agreed that loss of $p21^{Cip1}$ alone and/or combined with p53 accumulation predicted poor overall (Anttila et al., 1999; Costa et al., 1999; Schmider et al., 2000) and progression-free survival (Anttila et al., 1999; Ferrandina et al., 2000), but Sengupta et al. (2000), Shimizu et al. (1999) and Werness et al. (1999) insisted that $p21^{Cip1}$ failed to provide any significant information in this regard. Our data concur with those of the latter group of authors (Sengupta et al., 2000; Shimizu et al., 1999; Werness et al., 1999), although in the present series a lower survival probability was attributed to the loss of $p21^{Cip1}$ expression and to the $p21^{Cip1}$-negative/p53-positive phenotype in lower grade adenocarcinomas. The mechanisms by which loss of $p21^{Cip1}$ adversely affects survival are not known in detail. Perhaps the most obvious explanation is an abrogated cell-cycle block leading to less strict G1/S surveillance with facilitated transit into S phase. The absence, however, of a relationship between $p21^{Cip1}$ and proliferation does not quite support this scenario. $p21^{Cip1}$ may also mediate growth arrest by inhibiting further DNA synthesis, thus allowing the cells to continue their differentiation. Furthermore, the unfavorable prognostic effect of $p21^{Cip1}$ loss of expression (as well as that of $p21^{Cip1}$-negative/p53-positive phenotype) may reside in its relationship with stage and the volume of residual disease. The latter explanation seems to be entirely consistent with the loss of $p21^{Cip1}$ and of $p21^{Cip1}$/p53 predictive value once stage has entered the Cox's model. It is also conceivable that since $p21^{Cip1}$ may confer sensitivity to apoptosis, its overexpression will increase the susceptibility of ovarian cancer cells to platinum-based chemotherapy (Costa et al., 1999).

In conclusion, we present evidence that $p21^{Cip1}$ levels are related to various clinicopathological indicators of aggressiveness in ovarian adenocarcinomas and that different mechanisms may regulate its expression in LMP and adenocarcinomas. On the contrary, $p21^{Cip1}$ deregulation does not seem to participate in the pathogenesis of LMP tumors nor is it a

major player in cell proliferation of ovarian carcinomas. More importantly, although $p21^{Cip1}$, alone or combined with p53, is of prognostic significance in patients with lower grade adenocarcinomas, it does not appear to add to the information gained from traditional prognosticators. Prospective studies with a larger number of patients are warranted to validate this assumption.

References

Anttila M.A., Kosma V.-M., Hongxiu J., Puolakka J., Juhola M., Saarikoski S. and Syrjanen K. (1999). p21/WAF1 expression as related to p53, cell proliferation and prognosis in epithelial ovarian cancer. Br. J. Cancer 79, 1870-1888.

Barboule N., Mazars P., Baldin V., Vidal S., Jozan S., Martel P. and Valette A. (1995). Expression of $p21WAF1/^{Cip1}$ is heterogeneous and unrelated to proliferation index in human ovarian carcinoma. Int. J. Cancer 63, 611-615.

Cancer Committee of the International Federation of Gynecology and Obstetrics: Staging Announcement. (1989). Gynecol. Oncol. 25, 383-385.

Cordon-Cardo C. (1995) Mutations of cell cycle regulators. Biological and clinical implications for human neoplasia. Am. J. Pathol. 147, 545-560.

Costa M.J., Hansen C.L., Walls J.E. and Scudder S.A. (1999). Immunohistochemical markers of cell cycle control applied to ovarian and primary peritoneal surface epithelial neoplasms: $p21^{WAF/Cip1}$ predicts survival and good response to platinin-based chemotherapy. Hum. Pathol. 30, 640-647.

El-Deiry W.S., Tokino T., Velculescu V.E., Levy D.B., Parsons R., Trent J.M., Lin D., Mercer W.E., Kinzler K.W. and Vogelstein B. (1993). WAF1, a potent mediator of p53 tumor suppression. Cell 75, 817-825.

Ferrandina G., Stoler A., Fagotti A., Fanfani F., Sacco R., De Pasqua A., Mancuso S. and Scambia G. (2000). $p21^{WAF1/Cip1}$ protein expression in primary ovarian cancer. Int. J. Oncol. 17, 1231-1235.

Granna X. and Reddy E.O. (1995). Cell cycle control in mammalian cells. Role of cyclins, cyclin-dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). Oncogene 11, 211-219.

Hakes T.B. (1993). Prognostic factors in ovarian cancer. In: Cancer of the ovary. Markman M. and Hoskins W.J. (eds). Raven Press. New York. pp 115-131.

Johnson M., Dimitrov D., Vojta P.J., Barett J.C., Noda A., Pereira-Smith O.M. and Smith J.R. (1994). Evidence for a p53-independent pathway for upregulation of SDI1/Cip1/WAF1 p21 mRNA in human cells. Mol. Carcinog. 11, 59-64.

Jung J.M., Bruner M., Ruan S., Langford L.A., Kyritsis A.P., Kobayashi T., Levin V.A. and Zhang W. (1995). Increased level of $p21^{WAF1/Cip1}$ in human brain tumors. Oncogene 11, 2021-2026.

Korkolopoulou P., Konstantinidou A.-E., Thomas-Tsagli E., Christodoulou P., Kapralos P. and Davaris P. (2000). WAF1/p21 protein expression is an independent prognostic indicator in superficial and invasive bladder cancer. Appl. Immunohistochem. Mol. Morphol. 8, 285-292.

Korkolopoulou P., Kouzelis K., Christodoulou P., Papanikolaou A. and Thomas-Tsagli E. (1998). Expression of retinoblastoma gene product and p21 ($WAF1/^{Cip1}$) protein in gliomas: correlations with proliferation markers, p53 expression, and survival. Acta Neuropathol. 95, 617-624.

Korkolopoulou P., Vassilopoulos I., Konstantinidou A.-E., Patsouris E., Agapitos E. and Davaris P.S. (2002). The combined evaluation of $p27^{Kip1}$ and Ki-67 expression provides independent information on overall survival of ovarian carcinoma patients. Gynecol. Oncol. 85, 404-415.

Landis S.H., Murray T., Bolden S. and Wingo P.A. (1999). Cancer statistics. CA Cancer J. Clin. 49, 8-41.

Lukas J., Groshen S., Saffari B., Niu N., Reles A., Wen W.H., Felix J., Jones L.A., Hall F.L. and Press M.F. (1997). WAF,/Cip1 gene polymorphism and expression in carcinomas of the breast, ovary and endometrium. Am. J. Pathol. 150, 167-175.

Luo Y., Hurwitz J. and Massagué J. (1995). Cell cycle inhibition by independent CDK and PCNA binding domains in p21/Cip 1. Nature 375, 159-161.

Macleod K.F., Sherry N., Hannon G., Beach D., Tokino T., Kinzler K., Vogelstein B. and Jacks T. (1995). p53-dependent and independent expression of p21 during cell growth, differentiation and DNA damage. Genes Dev. 9, 935-944.

Masciullo V., Sgambato A., Pacilio C., Pucci B., Ferrandina G., Palazzo J., Carbone A., Cittadini A., Mancuso S., Scambia G. and Giordano A. (1999). Frequent loss of expression of the cyclin-dependent kinase inhibitor p27 in epithelial ovarian cancer. Cancer Res. 59, 3790-3794.

Palazzo J.P., Monzon F., Burke M., Hyslop T., Dunton C., Barusevicius A., Capuzzi D. and Kovatich A.J. (2000). Overexpression of $p^{21WAF1/Cip1}$ and MDM2 characterizes serous borderline ovarian tumors. Hum. Pathol. 31, 698-704.

Parker S.B., Eichele G., Zhang P., Rawls A., Sands A.T., Bradley A., Olson E.N., Harper J. W. and Elledge S.J. (1995). p53-independent expression of $p21^{Cip1}$ in muscle and other terminally differentiating cells. Science 267, 1024-1027.

Rey M.J., Fernandez P.L., Jares P., Munoz M., Nadal A., Peiro N., Nayach I., Mallofre C., Muntane J., Campo E., Estape J. and Cardesa A. (1998). $p21^{WAF1/Cip1}$ is associated with cyclin $D1^{CCND1}$ expression and tubular differentiation but is independent of p53 overexpression in human breast carcinoma. J. Pathol. 184, 265-271.

Russel P. (1994). Surface epithelial-stromal tumors of the ovary. In: Blaustein's Pathology of the Female Genital Tract. 4th ed. Kurman R.J. (eds). Springer-Verlag. New York. pp 705-782.

Schmider A., Gee C., Friedmann W., Lukas J.J., Press M.F., Lichtenegger W. and Reles A. (2000). p21 ($^{WAF1/Cip1}$) protein expression is associated with prolonged survival but not with p53 expression in epithelial ovarian carcinoma. Gynecol. Oncol. 77, 237-242.

Scully R. and Sobin L.H. (1987). Histologic typing of ovarian tumours. Arch. Pathol. Lab. Med. 111, 794-795.

Sengupta P.S., McGown A.T., Bajaj V., Blackhall F., Swindell R., Bromley M., Shanks J.H., Ward T., Buckley C.H., Reynolds K., Slade R.J. and Jayson G.S. (2000). p53 and related proteins in epithelial ovarian cancer. Eur. J. Cancer 36, 2317-2328.

Sherr C.J. (1996). Cancer cell cycles. Science 274, 1672-1677.

Shimizu M., Nikaido T., Toki T., Shiozawa T. and Fujii S. (1999). Clear cell carcinoma has an expression pattern of cell cycle regulatory molecules that is unique among ovarian adenocarcinomas. Cancer 85, 669-777.

Shiohara M., El-Deiry W.S., Wada M., Nakamaki T., Takeuchi S., Yang

R., Chen D.L., Vogelstein B and Koeffler H.P. (1994). Absence of WAF1 mutations in a variety of human malignancies. Blood 84, 3781-3784.

Skilling J.S., Sood A., Niemann T., Lager D.J. and Buller R.E. (1996). An abundance of p53 null mutations in ovarian carcinoma. Oncogene 13, 117-123.

Takeshima Y., Yamasaki M., Nishisaka T., Kitaguchi S. and Inai K. (1998). p21$^{WAF1/Cip1}$ expression in primary lung adenocarcinomas: heterogeneous expression in tumor tissues and correlation with p53 expression and proliferative activities. Carcinogenesis 19, 1755-1761.

Wan M., Cofer K.F. and Dubeau L. (1996). WAF1/Cip1 structural abnormalities do not contribute to cell cycle deregulation in ovarian cancer. Br. J. Cancer 73, 1398-1400.

Weinberg R.A. (1991). Tumor suppressor genes. Science 254, 1138-1146.

Werness B.A., Jobe J.S., DiCioccia R.A. and Piver M.S. (1997). Expression of the p53 induced tumor suppressor p21$^{Waf1/Cip1}$ in ovarian carcinomas: correlation with p53 and Ki-67 immunohistochemistry. Int. J. Gynecol. Pathol. 16, 149-155.

Werness B.A., Freedman A.N., Piver S., Romero-Gutierrez M. and Petro E. (1999). Prognostic significance of p53 and p21$^{waf1/Cip1}$ immunoreactivity in epithelial cancers of the ovary. Gynecol. Oncol. 75, 413-418.

Zhang H., Hannon G.J. and Beach D. (1994). p21 containing cyclin kinases exist in both active and inactive states. Genes Dev. 8, 1750-1758.

Zhang H., Xiong Y. and Beach D. (1993). Proliferating cell nuclear antigen and p21 are components of multiple cell cycle kinase complexes. Mol. Biol. Cell 4, 897-906.

Accepted March 12, 2003