Lack of Independent Prognostic Significance of p21 and p27 Expression in Advanced Ovarian Cancer: An Immunohistochemical Study

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ABSTRACT

The eukaryotic cell cycle is controlled by protein complexes consisting of cyclin-dependent kinases and cyclins. The cyclin-dependent kinases are in turn negatively regulated by a family of cyclin-dependent kinase inhibitors, comprising, among others, the p21 and p27 proteins. p21 and p27 have been shown to be of prognostic significance in a broad array of human tumors. Using immunohistochemistry, the frequency of expression and the possible prognostic and predictive significance of these proteins were examined in a series of 185 uniformly treated patients with stage III ovarian cancer. We found p21 to be overexpressed in 48% of cases. No significant correlation was found between the expression of p21 and p53 proteins (P = 0.273). A low level of p27 was demonstrated in 48.5% of cases. p21 overexpression correlated with lower Fédération Internationale des Gynécologistes et Obstétristes substage, lower patient age, and absence of ascites, but neither p21 nor p27 expression was of prognostic significance for the whole group of patients. Only a trend toward reduced survival (P = 0.092) was noticed for the small subgroup of patients (6%), whose tumors lacked p27 expression completely. A clear positive correlation could be found between p21 and p27 protein expression (P = 0.012). Despite the suggested role of the 21 and p27 proteins in determining drug sensitivity, they were not found to be predictive for response to chemotherapy, as assessed by second-look laparotomy in this large group of patients with advanced ovarian cancer.

INTRODUCTION

The CDKs, consisting of a catalytic subunit and a targeting (cyclin) subunit, are key regulators of cell cycle progression. Activation of the CDKs is dependent on their association with positive effectors, the cyclins, and on phosphorylation at a conserved threonine residue by the CDK-activating kinase (1–3). A number of small molecules have been identified that can bind to and inhibit the CDKs. These CDKs are grouped in two classes, based on their protein sequence homologies and putative CDK targets: the CIP/KIP family, including p21WAF1/Cip1, p27Kip1 and p57Kip2; and the INK4 family, including p15ink4b and p16ink4a (4).

p21 displays a selectivity toward inhibition of CDK/cyclin complexes that are involved in the transition from G1 to S phase. Here, p21 may act as an inhibitory buffer that sets the threshold cyclin levels required to activate this transition (5). It can also bind to proliferating cell nuclear antigen and block its ability to activate DNA polymerase δ, thereby inhibiting DNA replication (6). p21 was shown to be induced in response to DNA damage by wild-type but not mutant p53, leading to cell cycle arrest (7) and suggesting a role for p21 in DNA repair and apoptosis (8). The promoter for the p21 gene was demonstrated to contain two consensus p53-binding sites (7), and p21 is thought to be a major downstream effector of p53 (8). The p21 gene is inducible in a p53-independent manner by a variety of growth factors (9), an example of the latter being the p53-independent induction of p21 by transforming growth factor β in ovarian cancer cell lines (10). p21 can also be induced by the apoptosis-inducing anthracycline drug doxorubicin, through both p53-dependent and independent ways (8, 9). p21 acts as a tumor suppressor, and transfection of p21 cDNA was shown to suppress the growth of different cell lines (7). In contrast to the p53 gene, which is frequently mutated in human cancer, p21 gene mutations seem to be rare in a broad range of human malignancies, among them ovarian cancer (11).

The p27 protein associates with cyclin E-CDK2, cyclin A-CDK2, and cyclin D1-CDK4 complexes and prevents their activation or inhibits previously activated complexes. The removal of p27, through sequestration into cyclin D2-CDK complex, allows progression through the G1-S restriction point. p27 levels are high in arrested cells but low in proliferating cells (12, 13). It is up-regulated in vitro by transforming growth factor β, serum deprivation, contact inhibition, and rapamycin, an immunosuppressant that specifically inhibits progression from G1 to S phase, leading to cell cycle arrest (12–15). Transgenic p27 knockout mice have an increased body size, with a hyperplasia of thymus, pituitary, and adrenal glands and gonadal organs. They also display pituitary tumors and female sterility attributable to impaired ovarian follicle development (16, 17). These findings suggest an important role for p27 in the negative regulation of cell growth. In addition to CDK inhibition, p27 may have other functions. Recent evidence indicates that p27 overexpression induces apoptosis in several different human cancer cell lines, and the mechanism involved seems to be
different from p53-induced apoptosis (18). Mutations of the p27 gene, studied in a large number of human cancers, seem to be a very rare event (19), and regulation of p27 levels is thought to occur primarily at the posttranscriptional level (20).

Ovarian cancer is the most lethal of the gynecological malignancies and the fourth leading cause of cancer death in American women. About 75% of patients present in an advanced stage of the disease, and despite cytoreductive surgery and combination chemotherapy, most of them will ultimately succumb to the disease (21). We have shown previously that classical prognostic factors explaining differences in survival in advanced ovarian cancer comprise the amount of residual disease after first surgery, histological type, differentiation grade, presence of ascites, and age (22). The objective of this study was to examine the possible prognostic and predictive value of p21 and p27 in a large cohort of uniformly treated patients with stage III ovarian cancer, followed up over a long period of time.

**PATIENTS AND METHODS**

**Patients.** The patient cohort consisted of 185 patients consecutively included at the Department of Gynecologic Oncology at the Norwegian Radium Hospital in a multicenter trial on consolidation treatment after second-look laparotomy in stage III ovarian cancer patients. All patients were treated between January 1988 and May 1993. The median age was 54 years (range, 21–70). A staging laparotomy with an attempt at tumor debulking was performed in all patients, and postoperatively, a standard regimen containing cisplatin (50 mg/m² every 4 weeks) and epirubicin (50 mg/m² every 4 weeks) was used. To assess response, a second-look laparotomy was performed after four courses of chemotherapy in 149 of 185 patients. In 35 cases, no second-look laparotomy was performed because of clinically evident disease progression, and one patient preferred not to undergo the procedure. All patients were followed up until death or August 1998. Follow-up information was collected from the medical records, and no patients were lost for follow-up. Median follow-up time for patients still alive was 77

![Fig. 1](Image) Immunohistochemical analysis showing nuclear staining for p21 (×580).
months (range, 65–121). A detailed description of patient characteristics is given in Table 1.

**Immunohistochemistry.** Tumor tissue was obtained at the initial staging laparotomy in all patients. Sections from the paraffin-embedded blocks were immunostained using the biotin-streptavidin-peroxidase method (Supersensitive Immunodetection System, LP000-UL; Biogenex, San Ramon, CA) and OptiMax Plus Automated Cell Staining System (Biogenex). Deparaffinized sections were microwaved in 10 mM citrate buffer (pH 6.0) to unmask the epitopes and treated with 1% hydrogen peroxide for 10 min to block endogenous peroxidase. The sections were then incubated with monoclonal antibodies against p21 (Ab-1, 1:60, 1.7 μg IgG1/ml; Oncogene Science, Cambridge, MA) and p27 (K25020, 1:100, 2.5 μg IgG1/ml; Transduction Laboratories, Lexington, KY) for 30 min at room temperature. This was followed by incubation with biotin-labeled secondary antibody (1:40) and streptavidin:peroxidase (1:40) for 20 min each. Tissue was stained for 5 min with 0.05% 3',3-diaminobenzidine tetrahydrochloride freshly prepared in 0.05 m Tris buffer at pH 7.6 containing 0.024% H2O2 and then counterstained with hematoxylin, dehydrated, and mounted in Diatex. All of the dilutions of antibody, biotin-labeled secondary antibody, and streptavidin-peroxidase were made in PBS (pH 7.4) containing 1% BSA. All series included positive controls. Negative controls included substitution of the monoclonal antibody with mouse myeloma protein of the same subclass and concentration as the monoclonal antibody. All controls gave satisfactory results. Four semiquantitative classes were used to describe the number of positively stained cells: 0, none; 1+, <5% of tumor cells positive; 2+, between 5 and 50% of tumor cells positive; and 3+, > 50% of tumor cells positive. On the basis of the p21 and p27 expression pattern in normal ovarian

**Fig. 2** Immunohistochemical analysis showing one case with a high degree of p27 nuclear staining (a) and one case with a low degree of p27 nuclear staining (b). A high degree of nuclear staining was seen in the stromal cells (S; ×580).

**Fig. 3** Immunohistochemical analysis showing a single case with a high degree of nuclear staining for p27 in a well-differentiated area (a) and a low degree of p27 nuclear staining in a poorly differentiated area (b; ×580).
epithelial cells and previous experience (23), p21 protein expression was considered positive if any positive staining was seen in the tumor cells (≥1+), and p27 protein expression was classified as high when >50% of the tumor cells were immunoreactive. The review of the histological slides and immunohistochemical analyses were performed by two of the authors (R. H. and J. M. N.), unaware of the clinical data.

Statistical Analysis. Differences in proportions were evaluated by the χ² or Fisher’s Exact test, whichever was appropriate. Disease-free and corrected survival rates were calculated using the method of Kaplan and Meier (24). The log-rank test was used for univariate analysis, and a Cox proportional hazards regression model was used for multivariate evaluation of survival rates (25). In the multivariate analysis, a backward stepwise selection procedure was used. The hazard proportionality was verified by computing the log minus log against time. The Statistical Package for Social Science was used for the statistical analysis. Statistical significance was considered as P < 0.05.

RESULTS

p21 and p27 Protein Levels in Normal Ovarian and Stage III Ovarian Carcinoma Tissues. In samples of 10 normal ovaries, no p21 immunoreactivity was found in the epithelial cells. Nuclear p21 staining (Fig. 1) was detected in 88 of 185 carcinoma cases (48%). Sixty-seven (36%) had <5% of tumor cells staining, 20 (11%) between 5 and 50%, and only 1 case (0.5%) >50%. Nuclear expression of p27 protein was high (>50% of epithelial cells) in all normal ovaries and in 95 of 185 carcinomas (51.5%; Fig. 2a), whereas p27 immunostaining was low (<50% of tumor cells) in 90 of 185 carcinomas (48.5%; Fig. 2b). Eleven cases (6%) had no p27-positive cells, 62 cases (33.5%) had nuclear staining in between 5 and 50% of tumor cells, and 17 cases (9%) had <5% of cells stained. In two cases, well-differentiated areas of the tumor demonstrated a high level of p27 expression (Fig. 3a), whereas a low level of expression was found in the poorly differentiated areas (Fig. 3b).

Expression of p21 and p27 Protein in Relation to Clinicopathological Parameters. The frequencies of p21 and p27 immunostaining in relation to different clinicopathological parameters are shown in Table 1. There was a clear correlation between p21 positivity and lower FIGO stage (P = 0.024) and a borderline correlation between p21 positivity and lower age (P = 0.066) and absence of ascites (P = 0.061). No correlations could be found between p27 immunoreactivity and known clinicopathological parameters, regardless of the p27 cutoff level tested. However, a significant positive correlation was found between p21 and p27 protein expression levels (P = 0.012).

Relation between p21 and p53 Protein Expression. In a previous report (26), p53 protein expression was studied with immunohistochemistry in the same patient population. We were therefore able to make a comparison between p21 and p53 expression levels in the tumors. Forty-four cases (24%) were negative for both proteins, whereas 67 (36%) were positive for both. Twenty-one p53-negative cases (11%) showed p21 immunoreactivity, and 53 p53-positive cases (29%) were p21 negative. There was no significant correlation between the two variables (P = .273), strongly suggesting that there is a p53-independent regulation of p21 expression in advanced ovarian cancer.

p21 and p27 Expression in Relation to Patient Survival. At assessment on August 1, 1998, 19 patients (10%) were alive without evidence of disease, 10 (5.5%) were alive with disease, whereas 156 (84.5%) had died of ovarian cancer. The disease-related 5-year survival rate for the whole group was 25.4%. The median progression-free survival time was 13 months, and the median corrected survival time was 24 months. In univariate analysis, with the variables defined as in Table 1, age (P = 0.0192), FIGO stage (P < 0.0001), histological type (P = 0.0158), differentiation grade (P < 0.0001), ascites (P = 0.0003), and residual disease (P < 0.0001) were correlated to corrected survival. Neither p21 nor p27 protein expression reached statistical significance when their effect on survival was examined. Only when a complete loss of p27 protein expression was considered versus any degree of p27 expression was there a tendency toward statistical significance (P = 0.092). In multivariate analysis, only residual disease, histological type, degree of differentiation, and the presence of ascites were of independent prognostic significance (Table 2).

DISCUSSION

The frequency of p21 immunostaining in ovarian tumor tissues has been studied previously in a series of 44 ovarian carcinomas, 34% of which expressed p21 (27). In another one describing 17 cases, 75% were found positive (28). Lukas et al. (29) reported 80% of 53 specimens to be positive, but they also counted cases with only p21 immunostaining in benign stromal cell nuclei as positive. They reported a generally higher degree of p21 expression in the fibrous stroma surrounding the tumor. It is difficult to compare these numbers to the 48% p21 protein expression rate found in the present study, because information on the disease stage distribution of the cases was lacking in the other reports.

Our results are another example of the somewhat paradoxical situation of the overexpression of p21, a protein with the properties of a tumor suppressor, in cases of invasive cancer, as...
compared with normal epithelial cells. A similar finding was reported previously in other tumor types (30, 31). A possible explanation for this phenomenon could be that the increased expression of p21 may be the result of a feedback mechanism designed to halt proliferation. We were not able to demonstrate a correlation between p21 and p53 protein expression, suggesting that the expression level of p21 is regulated mainly by a p53-independent mechanism in advanced ovarian cancer. This is consistent with the findings in two other papers on p21 expression in ovarian cancer (27, 28), but in contrast to findings in other tumor types (32, 33), supporting the notion that the relative importance of p53-dependent and -independent pathways of p21 induction vary with tumor type. It should, however, be borne in mind that overexpression of p53 usually is the result of a p53 gene mutation. Because p53 mutations are frequent in stage III ovarian carcinoma (26), the association between the presence of native p53 and the expression of p21 may be difficult to detect in material such as ours.

p21 protein expression was found to be an independent unfavorable prognostic factor in esophageal (34) and breast (35) cancer but was of no independent prognostic significance in studies of malignant melanoma (36), pancreatic (33), and thyroid (37) cancer. Although in the present study correlations were observed with favorable prognostic factors such as lower FIGO substage, lower age, and absence of ascites, p21 was shown not to be of prognostic significance in advanced ovarian cancer.

We demonstrated low (<50% of cells) p27 expression in 48.5% of stage III ovarian cancers. No correlations could be demonstrated with known clinically important variables. However, we found a striking positive correlation between p21 and p27 protein expression ($P = 0.012$). This is difficult to explain because the major mode of regulation for p21 is transcriptional, whereas p27 seems to be regulated by a posttranscriptional mechanism through an ubiquitin-mediated proteasomal proteolysis (20), and the correlation should be addressed in future studies.

A low level of p27 expression has been shown to be an independent negative predictor of prognosis in several tumor types (38–40). Our results could only demonstrate a trend toward reduced survival ($P = 0.092$) in patients with tumors that had a complete loss of p27 protein expression, a rare event (6% of cases) in the cohort of patients studied. For the whole group, p27 had no independent prognostic significance. This is seemingly in contrast to the findings by Newcomb et al. (41), who found that p27 expression was of independent prognostic significance in a case control study where p27 was examined in 53 patients with stage IIc and III disease. The small sample size and the highly selected nature of the patient material (comparison of only patients surviving <2 years versus >5 years) in that study are likely explanations for the difference in results from the present report, a large cohort study of consecutively treated unselected patients.

We also examined the possible predictive value of CDKI expression by correlating the latter to response to chemotherapy, as assessed surgically after four courses of platinum/anthracyclin combination chemotherapy. Expression of p21 has been shown to confer resistance to apoptosis on differentiating myocytes (42), and high levels of constitutive p21 protein were an independent predictor of chemoresistance in acute myelogenous leukemia (43). High levels of p21 could inhibit the proliferation activity of the cells, thus rendering them less sensitive to drugs that preferentially kill cycling cells. With regard to p27, increasing evidence implicates this CDKI as a regulator of drug resistance in solid tumors (44). In human colon cancer cells, overexpression of p27 was linked to increased resistance to drugs like cisplatin, doxorubicin, and etoposide (45), substances also used in the treatment of ovarian cancer. In our material, however, no significant correlation could be found between the level of either p21 or p27 expression and response to chemotherapy.

In conclusion, immunohistochemical determination of p21 and p27 protein expression does not seem to contribute to a better prediction of prognosis and response to therapy in this large cohort of uniformly treated patients with advanced ovarian cancer.

ACKNOWLEDGMENTS

We thank Ellen Hellesylt, Mette Ingrud, and Liv Inger Häseth for technical assistance and Patricia Spaenhoven for secretarial help.

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