Subcellular Localization and Protein Levels of Cyclin-Dependent Kinase Inhibitor p27 Independently Predict for Survival in Epithelial Ovarian Cancer

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Abstract
Purpose: p27 protein is regarded as a valuable prognostic biomarker in cancer with a potential use as a molecular target. However, different methods of immunohistochemical assessment have yielded conflicting results. Here, we sought to determine the prognostic value of p27 in ovarian cancer using a novel method of compartmentalized in situ protein analysis.

Experimental Design: A tissue array composed of 150 advanced stage ovarian cancers uniformly treated, with surgical debulking followed by platinum-paclitaxel combination chemotherapy, was constructed. For evaluation of p27 protein expression, we used an immunofluorescence-based method of automated in situ quantitative measurement of protein analysis [automated quantitative analysis (AQUA)].

Results: The mean follow-up time of the patients was 34.3 months. Patients with low Fédération Internationale des Gynaecologistes et Obstétristes stage were more likely to have low nuclear p27 expression (P = 0.008). Low nuclear p27 expression was associated with improved 3-year overall survival (66% versus 20%, P = 0.0047) and disease-free survival (27% versus 12%, P = 0.022). In multivariable analysis, adjusting for well-characterized prognostic variables, low nuclear p27 expression level was the most significant prognostic factor for both disease-free and overall survival.

Conclusions: Our results indicate that quantitative assessment of nuclear p27 expression level by automated in situ quantitative analysis is a strong predictor for outcome in ovarian cancer.

Ovarian cancer is the fifth most common cancer in women. Despite the fact that it is highly curable if diagnosed early, cancer of the ovary kills more American women each year than all other gynecologic malignancies combined (1). There are no proven methods of prevention and it often is a rapidly fatal disease. If diagnosed and treated while the disease is confined to the ovary, the 5-year survival rate is 95%; however, only ~29% of all cases are detected at this early stage (1).

The current management of patients with advanced disease (stages III and IV) involves optimal surgical debulking followed by chemotherapy. The current standard chemotherapeutic approach for ovarian cancer patients includes taxane- and platinum-based regimens. Although this regimen is highly effective, 50% of women still die of the disease (1). Traditional clinicopathologic factors do not accurately classify patients in relation with prognosis. The only validated marker for ovarian cancer is CA-125, which is detectable in the serum of >80% of women with ovarian carcinomas (2). However, CA-125 is reliable only in monitoring response to treatment or disease recurrence and not as a diagnostic or prognostic marker (3). Therefore, considerable interest lies in identifying molecular prognostic indicators to guide treatment decisions.

The cell cycle regulators are particularly interesting because they are frequently altered in human cancer. p27Kip1 is a cyclin-dependent kinase (cdk) inhibitor that regulates cell cycle progression from G1 into S phase. In noncycling cells, p27 binds to cyclin E–cdk2 complexes and inhibits their activation (4). In proliferating cells, p27 binding to catalytically active cyclin D–cdk4/6 complexes results in p27 degradation and the subsequent release of cdk2 from inhibition (4). Thus, p27 helps to coordinate a balance between proliferation (when associated with cdk4/6) and arrest (when associated with cdk2; ref. 4). Antimitogenic signals or cell differentiation increase the amount of p27, initiating the timely exit of cells from the cell cycle (5). In contrast, mitogens and extracellular matrix adhesion signals promote p27 degradation so cells are actively engaged in the cell cycle (4). The p27Kip1 gene resides on chromosome 12p and is rarely mutated in human malignancies (6). Regulation of p27 protein level seems to occur primarily at the posttranslational level by ubiquitin-proteasome–dependent
degradation mechanisms (7). In ovarian cancer, studies report inconsistent associations between p27 protein levels and outcome. All of these studies used different methods of measuring p27 expression, with the majority using immunohistochemistry. Moreover, investigators used different antibodies at differing concentrations and different methods of immunohistochemical scoring.

Tissue microarrays are a useful tool for simultaneously studying specimens from hundreds of patients. This tool carries the inherent advantage of uniform handling of all specimens. Another positive feature associated with the use of tissue microarrays is the recently developed method of automated, quantitative analysis, which provides precise, reproducible, measurement of antigen levels, free of the subjectivity associated with pathologist-based scoring (8). Automated, quantitative analysis provides continuous output scores, as opposed to the arbitrary nominal scores obtained with pathologist-based “by-eye” scoring of 0, 1, 2, or 3, or “positive” and “negative.”

Here, we sought to determine whether p27 protein levels and expression pattern is associated with clinical outcome in a large cohort of uniformly treated patients with epithelial ovarian cancer using a novel in situ quantitative method of protein expression. Our study shows an independent prognostic value of p27 in determining patient outcome.

Materials and Methods

Patient population. Inclusion criteria were primary epithelial ovarian cancer patients [Fédération Internationale des Gynécologues et Obstétristres (FIGO) stages II, III, or IV] who underwent surgical resection in the Department of Gynecology of Alexandra University Hospital in Athens between 1996 and 2003 and treated postoperatively with carboplatin- and paclitaxel-based chemotherapy. Patients were subjected to staging laparotomy, including total abdominal hystereotomy, bilateral salpingo-oophorectomy, omentectomy, peritoneal biopsies, and surgical cytoreduction. Included patients had stage II, III, or IV disease according to the FIGO staging system. Grading was done by evaluation of tumor architecture, the amount of solid neoplastic areas, nucleus-cytoplasm ratio, and nuclear pleomorphism. The tumors were subdivided into three groups, well-differentiated (G1), moderately differentiated (G2), and poorly differentiated (G3) according to these criteria.

Our patient population was divided into two groups according to the extent of residual disease at first surgery, <2 and ≥2 cm. Chemotherapy was instituted 2 to 3 weeks after surgery. All patients received platinum-paclitaxel chemotherapy. Gynecologic examination, CA-125 assay, and radiological investigations, if necessary, were done monthly for the clinical assessment of response, which was recorded according to WHO criteria (9). Follow-up examinations were done every month.

Tissue microarray construction. A tissue microarray consisting of tumors from each patient in the cohort was constructed at the Yale University Tissue Microarray Facility. Following institutional review board approval, the tissue microarray was constructed as previously described (10), including 150 cases. Tissue cores 0.6 mm in size were placed on the recipient microarray block using a Tissue Microarrayer (Beecher Instrument, Silver Spring, MD). All tumors were represented with 2-fold redundancy. Previous studies have shown that the use of tissue microarrays containing one to two histospots provides a sufficiently representative sample for analysis by immunohistochrmistry (11–13). Addition of a duplicate histospot, although not necessary, does provide marginally improved reliability. The tissue microarray was then cut to yield 5 μm sections and placed on glass slides using an adhesive tape transfer system (Instrumedics, Inc., Hackensack, NJ) with UV cross-linking.

Immunohistochemistry. The experiment was done in duplicate (two microarray slides) to ensure accuracy. Tissue microarray slides were deparaffinized and stained as previously described. In brief, slides were deparaffinized with xylene followed by ethanol. Following rehydration in distilled water, antigen retrieval was accomplished by pressure cooking in 0.1 mol/L citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by incubating in 0.3% hydrogen peroxide in methanol for 30 minutes. Nonspecific antibody binding was then blocked with 0.3% bovine serum albumin for 30 minutes at room temperature. Following these steps, slides were incubated with primary antibody to p27 diluted at 1:50 (mouse monoclonal anti-p27; clone DCS-72.F6; Neomarkers, Fremont, CA) at 4°C overnight. This antibody has been extensively validated in previous studies using immunohistochemistry and Western blot analysis in normal and neoplastic tissue (14–18). Subsequently, slides were incubated with goat anti-mouse secondary antibody conjugated to a horseradish peroxidase–decorated dextran polymer backbone (Envision; DAKO Corp.) for 1 hour at room temperature. Tumor cells were identified by use of anticytokeratin antibody cocktail (rabbit anti-pancytokeratin antibody Z0622; DAKO) with subsequent goat anti-rabbit antibody conjugated to Alexa546 fluorophore (A11035, Molecular Probes). We added 4′,6-diamidino-2-phenylindole to visualize nuclei. Target (p27) molecules were visualized with a fluorescent chromogen (Cy5-tyramide, Perkin-Elmer Corp.). Cy5 (red) was used because its emission peak is well outside the green-orange spectrum of tissue autofluorescence. Slides were mounted with a polyvinyl alcohol–containing aqueous mounting media with antifade reagent (n-propyl gallate, Acros Organics).

Automated image acquisition and analysis. Automated image acquisition and analysis using automated quantitative analysis (AQUA) has been described previously (8). In brief, monochromatic, high-resolution (1,024 × 1,024 pixel; 0.5 μm) images were obtained of each histospot. We distinguished areas of tumor from stromal elements by creating a mask from the cytokeratin signal. 4′,6-Diamidino-2-phenylindole signal was used to identify nuclei and the cytokeratin signal was used to define cytoplasm. Overlapping pixels (to a 99% confidence interval) were excluded from both compartments. The p27 signal (AQUA score) was scored on a normalized scale of 1 to 255 expressed as pixel intensity divided by the target area. AQUA scores for each subcellular compartment (nuclear and cytoplasmic p27 signal) were recorded. Both microarray experiments were analyzed by AQUA and normalized AQUA scores for all tissue cores between the two experiments were averaged to obtain a mean normalized AQUA score for each tumor.

Statistical analysis. Individual histospots containing <10% tumor as assessed by mask area (automated) were excluded from further analysis. AQUA scores represent expression of a target protein on a continuous scale from 1 to 255. It is often useful to categorize continuous variable to stratify patients into high versus low categories. Several methods exist to determine a cut point, including biological determination, splitting at the median, and determination of the cut point that maximizes effect difference between groups. If the latter method (the so-called “optimal P value” approach) is used, a dramatic inflation of type I error rates can result (19). A recently developed program, X-Tile, allows determination of an optimal cut point while correcting for the use of minimum P value statistics (20). As the AQUA technology is new, there are no established cut points available for quantitative p27 expression. Therefore, for categorization of p27 expression levels, the X-Tile program was used to generate an optimal cut point. This approach has been successfully applied to AQUA data analysis (21, 22). Two methods of statistical correction for the use of minimal P value approach were used. First, the X-Tile program output includes calculation of a Monte Carlo P value for the optimal cut point generated. Cut points that yield Monte Carlo P values <0.05 are
considered robust and unlikely to represent type I error. Second, the Miller-Siegmann minimal $P$ value correction referenced by Altman et al. (19) was used. This approach is accepted in the statistical literature and is relatively unknown in the medical/biological research community (23, 24). Briefly, when making multiple comparisons to find the minimum $P$ value using the log-rank test, the false-positive rate (i.e., the percentage of times a marker that has no true prognostic value will be found to have a $P < 0.05$) can approach 40%. Altman’s statistical adjustment generates a minimum $P$ value corrected to yield a true false-positive rate of 5%. The corrected $P$ value ($P_{\text{corr}}$) is calculated as follows: $P_{\text{corr}} = \Phi(\xi) \left[ 1 - \left( 1 - \frac{1}{\Phi(\xi)} \right) \log(e) \right] \left[ \frac{1}{e} < 2 > / e < 2 > + 4 \Phi(\xi) / \xi \right]$, where $\Phi$ indicates the probability density function. $P_{\min}$ is the minimum $P$ value generated by evaluating multiple cut points. $\xi$ is the $\left( 1 - P_{\min} / 2 \right)$ quantile of the standard normal distribution. $e$ denotes the proportion of values excluded from consideration as an optimal cut point. Our calculations were done using an $\epsilon$ of 0.10. Disease-free survival and overall survival were subsequently assessed by Kaplan-Meier analysis with log rank for determining statistical significance and only $P_{\text{corr}}$ was reported. All survival analysis was done at 3-year cutoffs. Hazard ratios were assessed by multivariable Cox proportional hazards model.

Overall survival was defined as time from first day of chemotherapy to death from any cause. Disease-free survival was defined as time from first day of chemotherapy to the first of either death from any cause or disease progression (assessed by CA-125 increase and/or imaging studies). Performance status was dichotomized into 0 versus all others and histologic type into serous versus all others. 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. Our patient population was divided into two groups according to the extent of residual disease at first surgery; $<2$ and $\geq 2$ cm. Comparisons of p27 expression with FIGO stage and residual disease was made by Mantel-Haenszel $\chi^2$ test. Comparisons of p27 expression with performance status, histology, clinical response, and residual disease were made by Fisher’s exact test. Comparison of p27 expression status with age was made using Pearson correlation. All calculations and analyses were done with SPSS 12.0 for Windows (SPSS, Inc., Chicago IL).

Results

Clinical and pathologic variable analysis. One hundred fifty patients were included in the study. One hundred four patients had sufficient tissue for AQUA analysis. Mean follow-up time (range) for the cohort was 34.3 months. There were 4 (3.84%) FIGO stage II, 75 (72%) stage III, and 25 (24.16%) stage IV. Seventy-eight (75%) patients had tumors of serous histology. Initial histologic grade was 12 well differentiated (11.5%), 33 moderately differentiated (31.7%), 58 poorly differentiated (55%), and 1 not recorded. Seventy (67%) patients had an Eastern Cooperative Oncology Group performance status of 0. Following initial surgical debulking, residual disease by size was distributed as follows: 20 (19%) with $<2$ cm and 84 (81%) with $\geq 2$ cm. For clinical response to initial therapy, complete response or partial response was recorded in 63 (60.5%) patients, and stable disease/no response in 41 (39.5%) patients. Demographic and clinicopathologic variables for the cohort are summarized in Table 1.

Quantitative immunohistochemistry for p27 protein expression and generation of optimal cut point by X-Tile analysis. Of the 150 patients included in this study, 104 (69%) had sufficient tissue for analysis of p27 protein expression by AQUA. Tissues deemed insufficient had <10% tumor mask within the hotspot as represented on the tissue microarrays. As visualized by fluorescent immunohistochemistry, p27 displayed predominantly strong nuclear expression, whereas cytoplasmic p27 staining was generally weaker (Fig. 1A). Normalized AQUA scores for each subcellular compartment (cytoplasmic and nuclear) were represented on a 1 to 255 scale. p27 expression followed a skewed distribution as expected for a cancer tissue biomarker (Fig. 1B), with nuclear scores ranging from 3.6 to 255 AQUA units, whereas cytoplasmic scores ranged from 3.3 to 97.2 AQUA units. Using the X-Tile program, an optimal cut point for nuclear p27 expression was determined based on overall survival at 51.8 AQUA units, with a Monte Carlo $P$ value of 0.018 as determined by X-Tile. Monte Carlo $P$ values <0.05 indicate robust and valid cut point selection. Patients with nuclear p27 expression $<51.8$ were classified as low expressers (n = 78), and patients with nuclear p27 expression $>51.8$ were classified as high expressers ($n = 26$). For cytoplasmic p27, X-Tile analysis selected an optimal cut point at 32.7 AQUA units; however, the Monte Carlo $P = 0.30$ indicates lack of a valid cut point.

Association of p27 expression and clinicopathologic variables. Patients with high FIGO stage were more likely to have high nuclear p27 expression ($P = 0.008$). There was no association between nuclear or cytoplasmic p27 staining and clinical response, histologic type, histologic grade, performance status, or residual disease (Table 1).

Univariate survival analysis. Nuclear and cytoplasmic p27 expression status was evaluated for association with disease-free and overall survival using Kaplan-Meier survival analysis with log-rank statistic for determining significance. As use of an

| Variable | p27 Nuclear expression class | $n$ | Low | High | $P^*$ |
|----------|------------------------------|-----|-----|------|------|
| Age (y)  | range, 22–85                 |     |     |      | 1.000|
|          | median, 61                   |     |     |      |      |
| Grade    | Well differentiated           | 12  | 10  | 2    | 0.27 |
|          | Moderately differentiated     | 33  | 26  | 7    |      |
|          | Poorly differentiated         | 58  | 41  | 17   |      |
|          | Not recorded                  | 1   | 1   | 0    |      |
| Initial histology | Serous     | 78  | 57  | 21   | >0.5 |
|          | All others                    | 26  | 21  | 5    |      |
| Performance status | Zero     | 70  | 55  | 15   | 0.24 |
|          | All others                    | 34  | 23  | 11   |      |
| FIGO stage | II                   | 4   | 4   | 0    | 0.008*|
|          | III                          | 75  | 60  | 15   |      |
|          | IV                           | 25  | 14  | 11   |      |
| Residual disease (cm) | $<2$        | 20  | 17  | 3    | 0.39 |
|          | $\geq 2$                     | 84  | 61  | 23   |      |
| Clinical response | PR + CR  | 63  | 47  | 16   | >0.5 |
|          | All others                    | 41  | 31  | 10   |      |

Abbreviations: PR, partial remission; CR, complete remission. *Significant at the 0.05 level.
optimized cut point can result in increased type I error, the Miller-Siegmund correction method was applied to all Kaplan-Meier analyses. This analysis (Fig. 2) showed that low nuclear p27 expression is associated with increased 3-year disease-free and overall survival. Patients with low nuclear p27 expression had 27% disease-free and 66% overall survival compared with 12% and 20% for patients with high nuclear p27 tumors ($P_{\text{cor}} = 0.02$ and 0.0047, respectively). There was no significant association between cytoplasmic p27 expression status and disease-free or overall survival ($P_{\text{cor}} = 0.63$ and 0.33, respectively). These results are summarized in Table 2.

**Multivariable survival analysis.** Using the Cox proportional hazards model, we did multivariable analysis to assess the predictive value of p27 expression. Nuclear p27 expression by AQUA was analyzed for overall survival and disease-free survival. We included the following known prognostic variables in the regression model: age ($\leq 60$ versus $> 60$ years), histologic grade, FIGO stage, residual disease ($\leq 2$ versus $>2$ cm), response to chemotherapy (complete response/partial response versus all others), and initial histology (serous versus all others). Low nuclear p27 level was the only significant predictor variable of overall survival (hazard ratio, 3.2; 95% confidence interval, 1.7-6.2). For disease-free survival, nuclear p27 was the most significant prognostic factor (hazard ratio, 2.8; 95% confidence interval, 1.6-4.8) along with histologic grade: moderately differentiated (hazard ratio, 3.9; 95% confidence interval, 1.3-11.6). Results of multivariable survival analyses are summarized in Table 3.

Fig. 1. Protein expression of p27 was determined using AQUA analysis based on immunofluorescence. Digital images of each tumor spot were captured using Cy3 anticytokeratin antibody to generate a tumor mask. 4',6-Diamidino-2-phenylindole (DAPI) was used to visualize nuclei and Cy5 was used to visualize p27. A three-color merged image for each tumor is also shown (A). p27 expression followed a skewed distribution as expected for a cancer tissue biomarker (B), with nuclear scores ranging from 3.6 to 255 AQUA units, whereas cytoplasmic scores ranged from 3.3 to 97.2 AQUA units.

Fig. 2. Kaplan-Meier survival analysis for disease-free (A) and overall survival (B) by nuclear p27 expression level. Patients with high nuclear p27 AQUA score had worse disease-free and overall survival.
Our goal was to quantitatively assess expression of p27 on a cohort of ovarian cancer specimens in an objective, automated fashion, and to evaluate the association between p27 expression and clinical outcome. We showed nuclear p27 expression to be a robust predictor of disease-free and overall survival times in our cohort of patients. In multivariable analysis, nuclear p27 expression status retained its prognostic significance for disease-free and overall survival.

The relationship between p27 expression levels and prognosis is controversial. Loss of p27 expression has been found to be associated with increased risk of recurrence and poor survival. However, studies have also shown that increased expression of p27 can be a prognostic indicator of better outcome. In our study, we observed that patients with high nuclear p27 expression had significantly better survival outcomes compared to those with low expression.

### Table 2. Univariate 3-year survival analysis

| p27 Expression class | Mean survival (mo) | Cumulative percentage survival (95% confidence interval) | \( P^* \) |
|----------------------|--------------------|----------------------------------------------------------|----------|
| Disease-free survival |                    |                                                          |          |
| Low nuclear p27      | 21                 | 27.0% (16-38)                                            | 0.0224*  |
| High nuclear p27     | 13                 | 12.4% (0-25)                                             |          |
| Low cytoplasmic p27  | 20                 | 25.1% (15-35)                                            | 0.63     |
| High cytoplasmic p27 | 15                 | 14.7% (0-30)                                             |          |
| Overall survival     |                    |                                                          |          |
| Low nuclear p27      | 30                 | 65.7% (54-77)                                            | 0.0047*  |
| High nuclear p27     | 23                 | 19.5% (0-38)                                             |          |
| Low cytoplasmic p27  | 29                 | 62.8% (52-74)                                            | 0.33     |
| High cytoplasmic p27 | 27                 | 22.5% (0-44)                                             |          |

**NOTE:** \( P \) values given are for \( P_{cor} \) using the Miller-Siegmund method.

*Significant at the 0.05 level.

### Table 3. Multivariable 3-year survival analysis (Cox regression)

| Variable                              | Hazard ratio (95% confidence interval) | \( P \) |
|---------------------------------------|----------------------------------------|--------|
| Disease-free survival                 |                                        |        |
| FIGO stage                            |                                        |        |
| Stage II                              | (Reference category)                   |        |
| Stage III                             | 0.957 (0.2-4.4)                        | >0.5   |
| Stage IV                              | 2.066 (0.4-9.8)                        | 0.36   |
| Histologic grade                      |                                        |        |
| Well differentiated                    | (Reference category)                   |        |
| Moderately differentiated             | 3.934 (1.3-11.6)                       | 0.013* |
| Poorly differentiated                 | 2.179 (0.8-6.3)                        | 0.15   |
| Histology (serous/all others)         | 0.982 (0.6-1.7)                        | >0.5   |
| Residual disease (≤2/>2 cm)            | 2.060 (1.0-4.5)                        | >0.5   |
| Age (≤60/>60 y)                        | 1.722 (0.7-3.9)                        | >0.5   |
| Nuclear p27 expression (low/high)     | 2.757 (1.6-4.8)                        | <0.001*|
| Overall survival                      |                                        |        |
| FIGO stage                            | (Reference category)                   |        |
| Stage II                              | 1.002 (0.1-8.2)                        | >0.5   |
| Stage III                             | 1.599 (0.2-13.9)                       | >0.5   |
| Histologic grade                      |                                        |        |
| Well differentiated                    | (Reference category)                   |        |
| Moderately differentiated             | 3.155 (0.7-14.5)                       | 0.14   |
| Poorly differentiated                 | 2.238 (0.5-10.1)                       | 0.29   |
| Histology (serous/all others)         | 0.987 (0.5-2.1)                        | >0.5   |
| Residual disease (≤2/>2 cm)            | 1.240 (0.5-3.3)                        | >0.5   |
| Age (≤60/>60 y)                        | 1.169 (0.6-2.4)                        | >0.5   |
| Nuclear p27 expression (low/high)     | 3.217 (1.7-6.2)                        | <0.001*|

*Significant at the 0.05 level.
to be associated with worse prognosis in several cancers, including breast (25), prostate (26), bladder (27), hepatocellular (28), and colorectal (29) carcinoma. This is in contrast to reports that loss of p27 is associated with favorable outcome in pancreatic neuroendocrine (30), colon, esophageal, and endometrial (31) tumors. In ovarian cancer, conflicting data about the possible prognostic role of p27 status in advanced ovarian cancer patients also exists. Different methods of immunohistochemical grading probably account for these differences. Masciullo et al. (32) studied the association of p27 expression by immunohistochemistry in 99 patients with stages III and IV ovarian cancer with their clinical outcome. The authors found that loss of p27 expression (staining in <5% of cells) conferred poor prognosis. The authors scored for p27 staining regardless of cellular compartmentalization; both nuclear and cytoplasmic staining was included in the immunohistochemical score. Rosen et al., using the same cutoff (staining in <5% of cells) for nuclear staining, found that ovarian cancer patients with positive nuclear p27 staining had better disease-free survival. To the contrary, cytoplasmic p27 staining (any staining was considered positive) was associated with inferior outcome. Using a different cutoff value, Newcomb et al. (33), in a case-control study, reported that 78% of women with ovarian cancer who were long-term survivors had high levels of nuclear p27 (>50% of cells) compared with 17% of those with survival <2 years. Contrary to the findings of the aforementioned studies, Baekelandt et al. (34) found lack of prognostic significance of nuclear p27 (>50% versus <50% of cells) in a series of 185 patients with stage III ovarian cancer. In a similar fashion, Shimizu et al. (35) found no association between nuclear p27 levels and survival in patients with ovarian carcinoma.

To our knowledge, our study is the only one of its kind that evaluates the prognostic significance of p27 protein levels in different subcellular compartments using a novel quantitative in situ method of analysis. This method allows measurements of protein expression within subcellular compartments that results in a number directly proportional to the number of molecules expressed per unit area. Thus, we avoid biases introduced from the arbitrary cutoff points used in conventional immunohistochemistry studies while at the same time preserving spatial and morphologic information that techniques such as Western blotting lose.

Our finding that p27 overexpression predicts for unfavorable outcome seems paradoxical. However, besides loss of protein expression, nuclear overexpression of p27 has been proposed as an additional mechanism of functional p27 inactivation. Nuclear overexpression has been reported in uterine endometrioid carcinoma (31, 36), pancreatic endocrine neoplasms (30), and breast carcinoma (37). Nycum et al. (36) reported increased p27 staining with advanced grades of endometrial carcinoma. Watanabe et al. (31) found that high p27 expression was linked to cell proliferation and to higher grades of endometrioid adenocarcinoma. In a similar fashion, a positive correlation between p27 expression and Ki-67 expression was reported in the colon (38) and lung (39). Furthermore, in some rapidly proliferating breast cancer cells (40) and Burkitt’s lymphoma cells (41), a high level of p27 expression was seen. This up-regulation of p27 has been proposed to induce, rather than inhibit, cyclin-cdk signaling and, therefore, promote cell cycle progression (38, 42).

In summary, we show that measurement of nuclear p27 levels with automated in situ quantitative protein analysis in ovarian carcinomas is feasible and can provide important prognostic information.

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