Subcellular Localization of p27kip1 Expression Predicts Poor Prognosis in Human Ovarian Cancer

Daniel G. Rosen,1 Gong Yang,1 Kathy Qi Cai,1 Robert C. Bast Jr.,2 David M. Gershenson,3 Elvio G. Silva,1 and Jinsong Liu1
Departments of 1Pathology, 2Experimental Therapeutics, and 3Gynecologic Oncology, University of Texas M.D. Anderson Cancer Center, Houston, Texas

ABSTRACT

Purpose: The cyclin-dependent kinase inhibitor p27kip1 regulates cellular progression from G1 to S phase. Several studies have shown that loss of p27kip1 protein expression is associated with disease progression in various malignancies. The purpose of this study was to evaluate the subcellular localization of this cyclin-dependent kinase inhibitor in a large cohort of primary ovarian carcinomas and compare the results with clinicopathologic variables and overall survival.

Experimental Design: Subcellular localization of p27kip1 was first assessed by Western blotting in nuclear and cytoplasmic extract from 13 cases of ovarian carcinoma. Subcellular localization of the p27kip1 protein was evaluated using tissue microarrays containing 421 cases of ovarian carcinoma.

Results: The presence of p27kip1 in the cytoplasm regardless of the nuclear stain correlated strongly with late-stage disease (P < 0.03), extent of cytoreduction (P = 0.03), and shorter disease-specific survival (P < 0.0001).

Conclusion: Cytoplasmic localization of p27kip1 predicts poorer prognosis in ovarian carcinoma, particularly in late-stage disease.

INTRODUCTION

Human ovarian cancer is the fifth leading cause of death among women in the United States. The wide variability in response to treatment reflects the heterogeneity in tumor histotype and grade as well as the nonspecific nature of the symptoms associated with early-stage disease. The most consistently reported significant prognostic indicators for human ovarian cancer are disease stage, tumor grade, histotype, and extent of surgical cytoreduction. Although these factors are not always related to the biological behavior or aggressiveness of the disease, they are used as guidelines for selecting anticancer therapy. There is a wide spectrum of clinical behaviors from an excellent prognosis and a high likelihood of cure to those with rapid progression and poor prognosis irrespective of the clinical stage of the disease, most probably reflecting different biological properties of the tumors.

Cellular progression through the cell cycle is governed by cyclin-dependent kinase (cdk) that is regulated by phosphorylation, activated by binding of cyclins, and inhibited by cdk inhibitors. Based on their protein sequence homologies and putative cdk targets, cdk inhibitors belong to one of two families: the CIP/KIP family (p21Waf1/Cip1, p27kip1, and p57kip2), which inhibits a broad range of cyclin/cdk complexes and the INK4 family (p15Ink4b, p16Ink4a, p18Ink4c, and p19Ink4d), which inhibit mainly cdk4 and cdk6. The coordinated expression of cyclins, cdk, and cdk inhibitors is often deregulated in cancer (2). The cdk inhibitor p27kip1 regulates cellular progression from G1 to S phase. p27kip1 acts primarily by complexing with cyclins D1 and E, thereby inhibiting the function of these cdk. Several studies have shown that loss of p27kip1 protein expression is associated with disease progression in various malignancies (3–5) and with poor prognosis in prostate and colon cancer (4, 6). In breast cancer, the appearance of p27kip1 in the cytoplasm of tumor cells is associated with poor prognosis (3, 7). However, it is still controversial whether expression or loss of expression of p27kip1 has any prognostic significance in human ovarian cancer (7–11). In addition, the prognostic significance of subcellular localization has not been previously investigated. In this paper, we addressed this question by evaluating the subcellular localization of p27kip1 and its expression using tissue microarrays from 441 patients with ovarian cancer.

PATIENTS AND METHODS

Patients. Samples from women with primary epithelial ovarian cancer who had undergone initial surgery at the University of Texas M.D. Anderson Cancer Center between 1990 and 2001 were included in this study. A total of 441 correlating patients were identified. Follow-up information was updated through June 2003 by reviewing medical records and the U.S. Social Security Index. Demographic and survival data were entered into a comprehensive database created with Microsoft Access (version 97). Histopathologic diagnoses were based on WHO criteria (12–16); tumor grade based on Gynecologic Oncology Group criteria (17) and each case was assigned a disease stage according to the International Federation of Gynecology and Obstetrics system (18). Disease-specific survival rates were calculated as the percentage of subjects who survived with disease for a defined period, reported as time since diagnosis or treatment, and only deaths from the disease were counted. The extent of cytoreduction was defined as optimal if residual disease after surgery was smaller than 1 cm or...
suboptimal if residual disease was larger than 1 cm. (19, 20). Use of tissue blocks and chart review was approved by appropriate institutional committee.

**Nuclear and Cytoplasmic Extraction.** Ten cases of primary ovarian carcinoma were selected for Western blot analysis. Frozen sections were prepared and evaluated before processing to ensure correct sampling of the tumor and that at least 200 μg of pure tumor tissue (>80% tumor) had been collected from each case. The tissue samples were then homogenized in 500 μL of homogenizing buffer [10 mmol/L HEPES, 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 0.3 mmol/L sucrose, 0.1 mmol/L EGTA, 0.5 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1 μg/mL pepstatin, 5 μg/mL leupeptin, 0.15 unit/mL aprotinin, 1 mmol/L sodium fluoride, and 0.1% NP40 (pH 7.9)] using a Polytron microprobe set at “7” for 30 seconds on ice. The supernatant was transferred to another new tube and used for cytoplasmic fraction analyses. The pellet was dispersed by gentle mixing for 1 hour at room temperature using the same antibody against p27kip1 used for western blot analysis (1:100, clone 57, BD PharMingen, San Diego, CA), c-myc (Zymed, Los Angeles, CA), and to clinicopathologic information. Scoring discrepancies (11 of 13 cases, 3%), were resolved by a third pathologist. p27kip1 expression was graded semiquantitatively by the reviewers. The immunostained slides were reviewed by two pathologists, who followed the tissue microarray map to record a score for each sample. Each reviewer was blinded to the other’s assessment and to clinicopathologic information. Scoring discrepancies (11 cases, 3%), were resolved by a third pathologist. p27kip1 expression was graded semiquantitatively by the reviewers.

**Western Blotting.** Equal amounts of proteins (about 50 μg) were analyzed using standard methods for protein electrophoresis and transfer. The primary antibodies were to p27kip1 (clone 57, BD PharMingen, San Diego, CA), c-myc (Zymed Biotech, San Francisco, CA), β-actin (Sigma Chemicals, St Louis, MO). The secondary antibodies were against mouse or rabbit IgGs (Amersham Biosciences, Piscataway, NJ). The detection reagents were from an electrochemiluminescence kit (Amersham Biosciences). The adequacy of nuclear and cytoplasmic extracts was confirmed by using antibodies against c-myc (for nuclear samples) and β-actin (for cytoplasmic samples).

**Construction of the Tissue Microarrays.** Tissue blocks were stored under ambient conditions, at ~24°C. H&E-stained sections were reviewed by a pathologist to select representative areas of tumor from which to acquire cores for microarray analysis. Tissue microarray blocks were constructed by taking core samples from morphologically representative areas of paraffin-embedded tumor tissues and assembling them on a recipient paraffin block. This was done with a precision instrument (Beecher Instruments, Silver Spring, MD) that uses two separate core needles for punching the donor and recipient paraffin block. This was done with a precision instrument (Beecher Instruments, Silver Spring, MD) that uses two separate core needles for punching the donor and recipient paraffin block. The final tissue microarray consisted of six blocks, the first pair (blocks 1a and b) containing duplicates of 158 spots, the second pair (2a and b) containing duplicates of 164 spots, and the third pair containing duplicates of 119 spots. All samples were spaced 0.5 mm apart. Five-micrometer sections were obtained from the microarray and stained with H&E to confirm the presence of tumor and to assess the tumor histology. Tumor samples were randomly arranged on the blocks.

Sample tracking was based on coordinate positions for each tissue spot in the tissue microarray block; the spots were transferred onto tissue microarray slides for staining. This sample tracking system was linked to a Microsoft Access database containing demographic, clinicopathologic, and survival data on each patient, thereby allowing rapid links between histologic data and clinical features. The array was read according to the given tissue microarray map, each core was scored individually, and the results were presented as the mean of the two replicate core samples. Cases in which no tumor was found or no cores were available were excluded from the final data analysis.

**Immunohistochemical Analysis.** The tissue microarray slides were subjected to immunohistochemical staining as follows. After initial deparaffinization, endogenous peroxidase activity was blocked by using 0.3% H₂O₂. D Deparaffinized sections were microwaved in 10 mmol/L citrate buffer (pH 6.0) to unmask the epitopes. The slides were then incubated for 1 hour at room temperature using the same antibody against p27kip1 used for western blot analysis (1:100, clone 57, BD PharMingen), next with biotin-labeled secondary antibody for 20 minutes, and finally with a 1:40 solution of streptavidin-peroxidase for 20 minutes. Tissues were then stained for 5 minutes with 0.05% 3,3-diaminobenzidine tetrahydrochloride that had been freshly prepared in 0.05 mol/L Tris buffer at pH 7.6 containing 0.024% H₂O₂ and then counterstained with hematoxylin, dehydrated, and mounted. All of the dilutions of antibody, biotin-labeled secondary antibody, and streptavidin-peroxidase were made in PBS (pH 7.4) containing 1% bovine serum albumin. Colon carcinoma was used as a positive control. Negative controls were made by replacing the primary antibody with PBS. All controls gave satisfactory results.

The immunostained slides were reviewed by two pathologists, who followed the tissue microarray map to record a score for each sample. Each reviewer was blinded to the other’s assessment and to clinicopathologic information. Scoring discrepancies (11 cases, 3%), were resolved by a third pathologist. p27kip1 expression was graded semiquantitatively by the reviewers.
scoring system was based on the subcellular localization of the p27kip1 negative, nuclear, or cytoplasmic (21). The cytoplasmic
and nuclear stains were scored separately, not additively. Negative
staining was defined as absence of cytoplasmic stain and <5% of
positive nuclei. Cytoplasmic staining was scored on a three-point
system based on intensity: negative (no stain), weakly positive
(1+), and strongly positive (2+). When staining was present in the
nucleus but not in the cytoplasm, the sample was scored as
“nuclear staining only.” Nuclear staining was judged to be
positive if >5% nuclei in the sample were stained and negative if
<5% of the nuclei stained. Ten high-power fields were examined.
Normal ovarian epithelial cells were used as a comparison for
intensity and pattern of staining. The mean of the results from the
two replicate core samples from each tumor specimen was
considered for each case.

Statistical Analysis. Differences in proportions were
evaluated by the χ² or Fisher’s exact test as appropriate.
Kruskal-Wallis test was used to compare multiple independent
samples on the tissue microarray block containing normal ovary
and the different ovarian tumors. Also, differences in expression
levels between normal ovarian epithelial cells and the different
ovarian tumors was calculated using Mann-Whitney U test.
These results were adjusted for multiple comparisons and
considered statistically significant at the P < 0.01 level.
Disease-specific survival rates were calculated using the method
of Kaplan and Meier and compared by the log-rank test. Cox
proportional hazards regression models were used for multivari-
ate analysis of survival. Statistica software was used for the
statistical analysis (SAS Institute, SAS Language Reference,
version 8, SAS Institute, Inc., Cary, NC, 1999). Results were
considered statistically significant at the P < 0.05 level.

RESULTS

Patient Characteristics. The mean age of the 441
patients was 58.2 years (range, 20-96 years). With regard to
surgical disease stage, 36 patients (8.1%) had stage I disease, 32
(7.2%) had stage II disease, 291 (65.9%) had stage III disease,
and 82 (18.5%) had stage IV disease. The tumor histotype was
serous carcinoma in 336 patients (76.2%), endometrioid in 41
(9.3%), clear cell in 18 (4.1%), mixed malignant mullerian tumor
in 17 (3.9%), undifferentiated carcinoma in 12 (2.7), mucinous
in 10 (2.3%), and transitional cell carcinoma in 7 (1.6%). The
mean follow-up interval was 64 months (range, 1-120 months),
and the overall survival rate at 5 years was 38%.

Validation of p27kip1 Antibody. To determine the pro-
gnostic significance of p27kip1 according to its subcellular
localization, we first validated the antibody with Western
blotting in nuclear and cytoplasmic extracts from 13 randomly
selected cases of ovarian carcinoma (Fig. 1). Using the same
antibody, we immunostain whole sections of the same 13 cases
and compared the results with the Western blot. The Western
blots showed high expression of p27kip1 in the nucleus in three
cases (cases 2, 9, and 13) and in both the nucleus and cytoplasm
in the remaining 10 cases. Immunohistochemical analysis of
whole sections from the same 13 cases showed similar results,
the example photomicrographs of immunohistochemical staining
for case 2 (nuclear only) and case 8 in nuclear and cytoplasmic
extracts are shown in Fig. 1. In the five control cases (normal
ovarian surface epithelium), only nuclear staining for p27kip1
was observed (Fig. 1B, 3). Antibodies to c-myc (which is
present only in the nucleus) and β-actin (which is present only in
the cytoplasm) confirmed the proper preparation of the nuclear
and cytoplasmic extracts.

P27kip1 Subcellular Localization and Its Association
with Disease Stage, Tumor Grade, Patient’s Age, and Level
of Cytoreduction. Of all 441 cases identified, 421 (95.4%)
could be scored for p27kip1 staining; the remaining cases were
either lost during the sectioning procedure or did not meet the
criteria for inclusion. Results from immunostaining of the
microarrays, presented according to clinicopathologic character-
istics of the patients, are shown in Table 1. p27kip1 protein
was observed in the nucleus only in 122 cases (29%), in both
the nucleus and the cytoplasm in 240 cases (57%) and negative
expression in 59 of the cases (14%). With regard to the
cytoplasmic intensity staining, 96 cases showed weak intensity
and 144 cases strong intensity. No difference was found in the
association with clinical variables between the weakly and
strongly positive cytoplasmic staining groups (data not shown).
Therefore, additional analysis was done between negative,
cytoplasmic and nuclear localization.

Subcellular localization of p27kip1 was increasingly more
common in later-stage disease (P < 0.03). Concomitantly,
nuclear-only expression was more common in early-stage
disease (Table 1). No difference was observed in tumor grade,

Fig. 1B continued Immunohistochemical stains of the same 13 cases. 1, nuclear-only staining for p27kip1 corresponding to case 2 (black arrows); 2, cytoplasmic staining (black arrows) in a high-grade serous carcinoma (case 8); 3, normal ovarian surface epithelium showing only nuclear stain (black arrows, 40× magnification).
clinical stage. Among 355 late-stage disease patients, those with nuclear only expression of p27kip1 had a better overall survival than those with negative or cytoplasmic localization of the marker (P = 0.0002). A trend to better survival in cases with nuclear expression was also observed on early-stage disease group (P = 0.06).

We used a multivariate regression analysis based on the Cox proportional hazard model to test the independent value of each variable predicting overall survival among all patients and on those with late-stage disease. The estimated prognostic value of each variable in relation to overall survival is expressed as a P value. The variables used in Cox regression analysis are shown in Table 3. Subcellular localization of p27kip1 was an independent prognostic factor for poor survival among all patients (P = 0.0007; hazard ratio, 1.7; 95% confidence interval, 1.2-2.3) and also in those with late-stage disease (P = 0.001; hazard ratio, 1.6; 95% confidence interval, 1.2-2.2). Other independent prognostic factors associated with poor prognosis were International Federation of Gynecology and Obstetrics stage, age at diagnosis >60 years, and extent of cytoreduction.

DISCUSSION

In this large study of 421 patients with primary ovarian cancer with long-term follow-up available, we analyzed the prognostic significance of p27kip1 subcellular localization in terms of survival and its association with clinicopathologic variables. p27kip1 has been considered as a tumor suppressor gene and loss of its function has been associated with development of many types of human cancer. The tumor suppressor function of p27kip1 was first implicated in the context of cell cycle regulation (2). Recent studies showed that oncogenically activated kinase Akt/PKB can also phosphorylate p27kip1 at T157 inducing its relocalization to the cytoplasm (22, 23). Akt-mediated cytosolic accumulation of p27kip1 is critical for Akt mitogenic signaling. Akt-mediated exclusion of wild-type p27kip1 from the nuclear compartment results in activation of nuclear cdk2 and cell cycle progression, whereas a mutation at T157 confers resistance to Akt-mediated p27kip1 nuclear exclusion and impairs Akt-dependent rescue of p27kip1 induced cell cycle arrest (7). Hence, sufficient evidence has accumulated to suggest that cytoplasmic relocalization of p27kip1 might facilitate the tumor development. The presence of cytoplasmic p27kip1 (induced by phosphorylation at T157) has been shown to predict poor prognosis in breast cancer (22, 23).

### Table 1

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>p27kip1 Subcellular localization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(−)</td>
</tr>
<tr>
<td>Histotype</td>
<td></td>
</tr>
<tr>
<td>Serous carcinoma</td>
<td>40</td>
</tr>
<tr>
<td>Endometrioid adenocarcinoma</td>
<td>7</td>
</tr>
<tr>
<td>Clear cell carcinoma</td>
<td>3</td>
</tr>
<tr>
<td>Mixed malignant millerian tumor</td>
<td>1</td>
</tr>
<tr>
<td>Mucinous adenocarcinoma</td>
<td>1</td>
</tr>
<tr>
<td>Transitional cell carcinoma</td>
<td>2</td>
</tr>
<tr>
<td>Subtotals</td>
<td>59</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Localization</th>
<th>Mean survival</th>
<th>Survival rate at 2 y</th>
<th>Survival rate at 5 y</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear only</td>
<td>122</td>
<td>41.4</td>
<td>68.4</td>
<td>30.1</td>
</tr>
<tr>
<td>Cytoplasmic</td>
<td>240</td>
<td>38.0</td>
<td>56.4</td>
<td>32.6</td>
</tr>
<tr>
<td>Nuclear only</td>
<td>59</td>
<td>55.3</td>
<td>77.5</td>
<td>57.9</td>
</tr>
</tbody>
</table>

*P = 0.0001

**Table 1 Localization of p27kip1 in terms of patient and tumor characteristics**

The table provides a summary of the subcellular localization of p27kip1 in relation to various patient and tumor characteristics. The localization is categorized into three groups: nuclear (N), cytoplasmic (C), and negative (−), with subtotals for each category. The table includes histotype, tumor grade, FIGO disease stage, extent of cytoreduction, patient's age at time of diagnosis, and FIGO disease stage. Statistical significance is indicated by *P* values calculated using the chi-squared test of independence.

**Table 2 p27kip1 localization and overall disease-specific survival**

The table shows the mean survival and survival rates at 2 and 5 years for patients with p27kip1 localization in different categories: nuclear only, cytoplasmic, and without nuclear staining. The survival rates are presented as percentages, and statistical significance is indicated by *P* values derived from the Kaplan-Meier analysis.
Other studies have also analyzed the relationship of p27kip1 in ovarian carcinoma with survival using different scoring systems to quantify its expression (8–11, 24, 25). In two studies of 66 and 99 cases and scoring all immunoreactive cells regardless the subcellular localization, Masciullo et al. (8, 9) described that p27kip1 is an independent prognostic factor of disease progression and survival; however, no correlation with other clinicopathologic variables was found. Scoring the frequency of only nuclear immunopositive cells in a series of 54 patients (30 long-term and 24 short-term survivors), Newcomb et al. (10) also reached to similar conclusions. Hurteau et al. (21) found that decreased nuclear staining of p27kip1 was associated with poor survival in some epithelial ovarian cancers. In another study including 79 cases of ovarian carcinoma, Shigemasa et al. did not find an effect on prognosis. However, they did find a prognostic significance when only the serous carcinomas cases were studied (24). The only other study that showed lack of prognostic significance is the one from Baekelandt et al. (11). These diverse results probably reflect differences in the number of subjects, study design, and protein quantification methods among these studies, in particular specificity of antibodies.

In this study, we first validate the specificity of the antibodies used to detect the expression of subcellular localization on immunohistochemistry. Using this validated antibody, we have shown that the nuclear only expression of p27kip1 is a favorable prognostic marker (21). However, the most interesting results from our study, and one that differs from all previous ones, was the association between cytoplasmic localization of p27kip1 and prognosis in human ovarian cancer. The presence of p27kip1 in the cytoplasm and regardless of the nuclear expression correlated with higher International Federation of Gynecology and Obstetrics disease stage (\( P < 0.03 \)), with extent of cytoreduction (\( P < 0.03 \)), and with shorter disease survival (\( P < 0.0001 \)). By analyzing the subcellular localization
of this cdk inhibitor, we were able to identify a subset of patients with particularly poor outcome. Using the Cox proportional hazard model of factors influencing survival, we could also show the prognostic independence of p27kip1 in both groups, one including all the patients and in the other only those with late-stage disease. However, the underlying mechanism of why patients with negative and cytoplasmic relocalization of p27kip1 have poor prognostic significance is not clear and will require further investigation.

In conclusion, p27kip1 subcellular location in the cytoplasm was independently associated with poorer survival among women with ovarian carcinoma, particularly for those with late-stage disease and regardless of tumor histotype.

REFERENCES

Clinical Cancer Research

Subcellular Localization of p27kip1 Expression Predicts Poor Prognosis in Human Ovarian Cancer

Daniel G. Rosen, Gong Yang, Kathy Qi Cai, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/11/2/632

Cited articles
This article cites 24 articles, 5 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/11/2/632.full.html#ref-list-1

Citing articles
This article has been cited by 15 HighWire-hosted articles. Access the articles at:
/content/11/2/632.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.