Frequent Loss of Expression of the Cyclin-dependent Kinase Inhibitor p27 in Epithelial Ovarian Cancer

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ABSTRACT

p27Kip1 is a member of the Cip1/Kip1 family of cyclin-dependent kinase inhibitors and is a potential tumor suppressor gene. Low levels of p27 are associated with poor prognosis in a variety of tumors, including breast, colon, prostate, and lung carcinomas.

In the present study, p27 protein expression was investigated by immunohistochemistry and Western blot analysis in a series of 82 epithelial ovarian tumors [16 classified as low malignant potential (LMP) and 66 classified as primary ovarian adenocarcinomas]. Immunohistochemical analysis revealed frequent loss of p27 expression in primary ovarian adenocarcinomas (33%), with respect to LMP tumors (6%; \( P = 0.0009 \)). In addition to nuclear staining, cytoplasmic localization of p27 was noted in 45 (55%) of 82 cases. p27 levels inversely correlated with cdk2 kinase activity in a representative subset of tumors. When the clinical outcome of the patients was evaluated in relationship to p27 status, we observed a significant correlation between presence of p27 staining and a longer time to progression (\( P = 0.032 \) by log-rank test).

These data indicate that loss of p27 is a frequent event in ovarian carcinomas as compared with LMP tumors, suggesting that these tumor types may have different pathogenesis. p27 levels may also represent a useful prognostic marker for predicting disease recurrence in primary ovarian carcinomas.

INTRODUCTION

The eukaryotic cell cycle is controlled by protein kinase complexes composed of cyclins and Cdkks. The activity of Cdkks is regulated by binding of positive effectors, the cyclins, and by association-dissociation of inhibitory subunits, designated CKIs (1). Two families of CKIs have been identified. INK4 family members p15INK4B, p16INK4A, p18INK4C, and p19INK4D bind to and inhibit cyclin-D-dependent kinases cdk4 and cdk6 (2). KIP family members, including p21CIP1, p27KIP1, and p57KIP2, preferentially inhibit cdk2 (1).

Cyclins, cdkks, and CKIs are frequently altered in human cancer (3). p27Kip1 is a CKI that regulates progression from G1 into S phase by inhibiting a variety of cyclin-cdk complexes, including cyclin D-cdk4, cyclin E-cdk2 and cyclin A-cdk2. p27 appears to play a role in both cell growth and differentiation, because its ectopic overexpression induces differentiation of some cell lines (4).

The p27Kip1 gene is located on chromosome 12p and, unlike the genes encoding INK4 family members, is rarely affected by structural alterations in human malignancies (5). However, levels of p27 in human cancer seem to be regulated at the posttranscriptional level by ubiquitin-proteasome-dependent degradation mechanisms (6).

Several studies have demonstrated that loss of p27Kip1 protein, as assessed by immunohistochemistry, is a negative prognostic marker in some malignancies, including breast (7, 8), colon (9), lung (10), prostate cancer (11), and malignant lymphomas (12). In addition, reduced expression of p27Kip1 correlates with tumor grade in prostate (13) and colon cancer (14).

Ovarian cancer is the most common gynecological malignancy causing fatality in western countries and the fifth leading cause of female cancer death. Traditional clinicopathological criteria used to predict clinical outcome are largely inadequate. Despite progress in surgical and chemotherapeutic treatment, the 5-year survival rate for all stages of ovarian cancer has remained constant at 39% over the last 30 years (15). Thus, great benefit is likely to result from the characterization of additional prognostic factors, more closely related to tumor cell biology. These biological factors may offer novel approaches to the identification of groups of patients that could benefit from more aggressive therapy.

It is conceivable that most ovarian cancers occur as a result of acquired alterations in oncogenes and tumor suppressor genes regulating signal transduction pathways involved in cell proliferation and differentiation, as well as cell cycle control (16). Studies have shown that alterations in the p53 (17), HER-2/neu (18) and ras genes (19) are associated with poor prognosis in ovarian cancer. CKIs are also frequently altered in ovarian cancer; loss of p21 (20) and p16 (21) expression has been reported in 20–25% of ovarian carcinomas.

In this study, the expression of p27Kip1 protein was investigated by immunohistochemistry on tissue samples from 82 patients affected by epithelial ovarian tumors, and the correlation with patient outcome was evaluated.

MATERIALS AND METHODS

Patients and Tumor Specimens. Eighty-two epithelial ovarian tumor specimens were obtained from patients who underwent surgical resection in the Department of Gynecology of the Catholic University of Rome. Sixty-six specimens were classified as primary malignant tumors, whereas 16 specimens were classified as LMP tumors. Histological classification of tumors was carried out according to the WHO system, and tumors were graded as well (G1), moderately (G2), and poorly differentiated (G3). Clinical stages of disease were established according to the FIGO staging system.

After surgical resection, each tumor specimen was divided into two portions: one portion was instantly frozen for protein extraction, whereas the second portion was immediately formalin-fixed and then paraffin-embedded for routine and immunohistochemical investigation. The histological analysis of serial frozen sections confirmed the presence of a homogeneous lymphocytic component in the samples analyzed. Therefore, to take into consideration the lymphocytic contamination of tumor samples, five serial frozen sections were prepared from tissues where the other portion was used for Western analysis and each section was evaluated for the percent of lymphocytes over total cell number by light microscopy evaluation of H&E-stained slides.

Immunohistochemistry and Specificity of Immunostaining. Immunohistochemistry was performed as described previously (13). Briefly, the poly-

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3 The abbreviations used are: Cdk, cyclin-dependent kinase; CKI, Cdk inhibitor; LMP, low malignant potential; KIP, kinase inhibitor protein.
clonal antibody to p27Kip1 (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated overnight with tissue sections at 1:500 dilution. Specificity of p27 Kip1 staining was assessed by preabsorption with the peptide used to generate it. The pattern of staining seen with the p27 Kip1 polyclonal antibody was confirmed on duplicate slides using a monoclonal antibody (PharMingen, San Diego, CA). The strong positive immunostaining of lymphocytes, in the sections examined, represented an internal positive control for preservation of the p27 antigenicity in tissues. For negative control, PBS was substituted for the primary antibody.

**p27 Scoring.** Because the preabsorption test abolished both nuclear and cytoplasmic staining, cells were scored for p27 staining regardless of cellular compartmentalization. All immunoreactive cells were considered positive. Three pathologists (A. Ca., A. S., and J. P.) separately evaluated p27 staining in a coded manner: score 1, <5% of positive cells; score 2, 5–50% of positive cells; score 3, >50% of positive cells. Subsequent statistical analysis of the data, however, showed no advantage in separating groups 2 and 3; consequently, protein levels were classified as positive (staining in >5% of cells) or negative (staining in <5% of cells). At least 20 high-power fields were chosen randomly, and 2000 cells were counted.

**Immunoblotting and Kinase Assay.** Each frozen ovarian cancer tissue sample (1 g; 10 representative samples) was sectioned and quickly homogenized in 250 mM NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 0.1% (v/v) Triton X-100, 1 mM phenylmethylsulfonl fluoride, 50 mM NaF, 0.5 mM Na3VO4, 10 mg/ml leupeptin, and 50 mg/ml aprotinin. Conditions for immu-
nobilting and histone H1 kinase assay have been described previously (10, 22).

Statistical Analysis. Fisher’s exact test for proportion and the χ² test were used to analyze the distribution of p27-positive cases according to clinicopathological characteristics. All medians and life tables were computed using the product-limit estimate, and the curves were examined by the log-rank test. Time to progression was calculated from the date of first surgery to the date of clinical or pathological progression. Survival analyses were carried out using SOLO Statistical Software (BMDP Statistical Software Inc).

RESULTS

p27 Protein Expression in Epithelial Ovarian Cancer. The expression of p27 in ovarian cancer was determined by immunohistochemical analysis. Immunoreactivity for p27 was found in both normal and neoplastic tissues. Strong p27 expression was detected in ovarian surface epithelium and stromal cells of normal ovarian tissue. p27 immunostaining was mostly nuclear; however, weak cytoplasmic staining was also observed (Fig. 1, A and B). A total of 82 epithelial ovarian tumors were evaluated. Sixteen tumors were found to be LMP and were classified as mucinous (n = 9), serous (n = 4), or endometroid (n = 3). LMP tumors exhibited focal immunostaining for p27 and were scored as positive in 15 of 16 cases (94%); in one case, p27 expression was only nuclear (Fig. 1, C and D); in five cases, p27 was localized both in the nucleus and cytoplasm (Fig. 1, E and F), whereas nine cases had exclusive cytoplasmic localization.

Primary ovarian adenocarcinomas expressed p27 in 33 of 66 cases (50%), whereas absence of p27 protein expression (<5% positive cells) was observed in the remaining 50% of cases. In contrast to normal epithelium, where p27 was mostly localized in the nuclei, ovarian adenocarcinomas showed cytoplasmic staining of p27, with (15 cases; Fig. 2, A and B) or without (16 cases; Fig. 2, C and D) concomitant nuclear staining. In two cases, p27 was exclusively present in the nuclei of a focal area of the tumor (Fig. 2, E and F). In most cases, p27 staining intensity was consistently lower in primary tumors compared with the normal ovarian epithelium. A statistically significant difference was found in p27 expression of LMP tumors with respect to ovarian carcinomas (P = 0.0009; Table 1).

We decided to investigate by Western blot analysis whether similar p27 protein expression was present in a subset of 10 representative primary ovarian tumors. As an internal control, the blots included total lysate from the ovarian cancer cell line OVCAR-2, which was shown to express p27 at levels comparable with those of immortalized human ovarian surface epithelium (Fig. 3). Samples showing a p27 signal at least equal to the OVCAR-2 control line were scored as positive. To estimate what proportion of the signal was contributed by the normal cellular component of the tumor, we evaluated the lymphocyte component of serial sections of the samples. In this experimental subset, p27 protein was expressed at different levels and showed a good correlation with p27 immunostaining when the percentage of lymphocytes present in the corresponding tumor section was taken into account (Fig. 4A). In particular, 4 of the 10 ovarian adenocarcinomas showed undetectable levels of p27 protein. To verify that the same amount of total protein was present in each lysate, the same blot was reprobed with a polyclonal antibody against ERK 1 (Santa Cruz Biotechnology).

Because of the role of p27 in controlling cell proliferation through the inhibition of cdk2, we examined cdk2-associated histone H1 kinase activity in the same primary ovarian carcinoma lysates. Cdk2 activity was detected in all of the samples and inversely correlated with p27 levels (Fig. 4B), although these measures should be considered only approximate due to the heterogeneous nature of the tumor samples.

Correlation of p27Kip1 Expression with Clinicopathological Parameters and Survival Analysis. We investigated the relationship between the expression of p27 and a series of clinicopathological parameters (age, stage, grading, ascites, residual tumor, response to chemotherapy) in the 66 primary ovarian tumors. LMP tumors were excluded from the analysis due to their different prognostical outcome compared with primary ovarian carcinomas. p27 staining did not correlate with any of the clinicopathological parameters examined (data not shown).

Follow-up data were available for 66 patients (median follow-up, 29 months; range, 2–145 months). During the follow-up period, progression of disease was observed in 34 patients. Fig. 5 shows the time to progression curve in relation to p27 status. The median time

Table 1 Distribution of p27 immunostaining by ovarian tumor type

<table>
<thead>
<tr>
<th>p27 staining</th>
<th>LMP (n = 16)</th>
<th>Primary ovarian carcinoma (n = 66)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive (≥5%)</td>
<td>15 (94%)</td>
<td>33 (50%)</td>
<td>0.0009</td>
</tr>
<tr>
<td>Negative (&lt;5%)</td>
<td>1 (6%)</td>
<td>33 (50%)</td>
<td></td>
</tr>
</tbody>
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Fig. 3. Western blot analysis of p27 levels in OVCAR-2 ovarian cancer cell line and human ovarian surface epithelium (LK). Samples express comparable levels of p27 protein.

Fig. 4. A, p27 differential expression in primary ovarian carcinomas was assayed by Western blot. B, Cdk2 immunoprecipitates from the same primary tumor lysates were assayed for associated histone H1 kinase activity. Radioactivity in histone H1 bands is shown. When p27 staining was low in tumor cells, cdk2 activity was high. In contrast, tumors with high p27 staining showed low kinase activity.

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hyperplasia and malignancy of tissues (1, 3). Moreover, recent studies have demonstrated that inactivation of CKIs has been associated with increased Cdk2 activity. It has been hypothesized that the frequent association of cyclin D1 overexpression with decreased p27 levels is a consequence of the disruption of a homeostatic feedback mechanism in a subset of unfavorable prognostic tumors (14). Thus, it is conceivable that in ovarian cancer p27 loss integrates the effect of one or more cell cycle regulators of the same pathway, possibly including cyclin E and D1.

Subcellular compartmentalization of p27 was previously observed in normal prostate tissue, dysplastic Barrett’s epithelium, and colorectal (9, 14), prostate (13), and esophageal cancer (32). It is interesting to note that p27 expression in ovarian cancer is frequently cytoplasmic (with or without concomitant nuclear staining). The mechanism responsible for this phenomenon and its biological significance in ovarian cancer cells is still unknown. However, it is noteworthy that cytoplasmic displacement of p27 has been linked to loss of tumor suppressor genes, such as tuberin (33), or to binding to a transcriptional activator, such as Jab1 (34).

Loss of p27 expression did not correlate with any of the clinico-pathological parameters used to predict clinical outcome, but it was associated with short time to progression. Interestingly, this association was also retained after the exclusion of stage I and stage II tumors (i.e., tumors with predictable long-term survival), further supporting the hypothesis that loss of p27 confers a more aggressive phenotype to tumor cells (7–11) and, therefore, might play an important role in the development of ovarian cancer. The lack of association with any clinico-pathological parameter may suggest that p27 is independent in assessing the risk of progression. Despite the small population analyzed in this study, the validity of this observation is supported by previous studies in breast (7), colorectal (9), lung (28), and prostate (29, 35) cancer, in which the expression of p27 protein was an independent significant predictor of poor disease-free survival. Studies are ongoing to assess this hypothesis on a larger series of patients with a longer follow-up. During the preparation of this manuscript, another study appeared in which the expression of p27 was investigated in a series of primary ovarian carcinomas. In this study (36), no correlation was found between p27 expression and survival of the patients. However, the smaller number of cases analyzed and the choice of a different cutoff may account for the difference with our results.

In summary, our findings show that loss of p27 is a frequent event in primary ovarian adenocarcinomas and that p27 expression reflects its function as a cdk2 inhibitor. We also present evidence of differential p27 staining in LMP and primary ovarian carcinomas, suggesting a different pathogenesis for these two tumor types. Furthermore, p27 expression may be an effective indicator of clinical behavior in primary ovarian adenocarcinomas and, therefore, a putative new prognostic marker for ovarian cancer.

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REFERENCES

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