Frequent Loss of pRb2/p130 in Human Ovarian Carcinoma


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ABSTRACT

Purpose: RB2/p130, a member of the retinoblastoma gene family, maps to human chromosome 16q12.2, a region in which deletions have been found in several human neoplasms including breast, prostatic, and ovarian carcinoma. We sought to evaluate pRb2/p130 protein expression and function in ovarian carcinoma.

Experimental Design: pRb2/p130 expression was detected by immunohistochemical and Western blot analyses in 45 primary ovarian carcinoma samples.

Results: Immunohistochemical analysis revealed loss or decrease of pRb2/p130 expression in 18 cases (40%), pRb2/p130 expression was mostly nuclear and inversely correlated to the tumor grade (P < 0.05). Western blot analysis correlated with immunohistochemical expression. Reverse transcription-PCR followed by Southern blot analysis was performed on a representative set of 20 ovarian carcinomas. RB2/p130 mRNA levels were consistent with protein expression. We found a significant increase in the percentage of G1-phase-arrested cells in CAOV3 and A2780 ovarian carcinoma cell lines after transduction with an adenovirus carrying the RB2/p130 gene (Ad-CMV-RB2/p130).

Conclusions: These data indicate that loss or decrease of pRb2/p130 expression is a frequent event in ovarian carcinoma and is regulated mostly at the transcriptional level. Moreover, pRb2/p130 overexpression is able to arrest cell growth in ovarian carcinoma cells, suggesting the putative role of pRb2/p130 as a tumor suppressor in this malignancy.

INTRODUCTION

RB2/p130, together with RB/p105 and p107, forms the retinoblastoma (Rb) gene family (1, 2). The proteins encoded by these genes play a pivotal role as negative regulators of cell proliferation, although the growth arrest mediated by each of them is not identical (3). Recent reports have identified pRb2/p130 as the Rb family member most responsible for the control of cell cycle progression. In fact, pRb2/p130 in association with E2F4 is the most abundant Rb family-E2F complex found in G0-G1 resting cells or in G0 quiescent cells and is thought to help to maintain a state of transcriptional silence (4, 5). Progression of the cells through G1 and S phase requires inactivation of Rb proteins by phosphorylation. pRb2/p130 activity is also regulated by intracellular localization: the hypophosphorylated form of pRb2/p130 is nuclear and typical of cells in G0 phase; whereas the hyperphosphorylated form is cytoplasmic and typical of cells progressing into G1 phase (6). pRb2/p130 also shows an important kinase inhibitory activity, suggesting that part of its growth-inhibitory function is mediated by inhibiting important cell cycle kinases (7, 8).

The RB2/p130 gene consists of 22 exons, spanning over 50 kb of genomic DNA (9), and maps to human chromosome 16q12.2, an area in which deletions or loss of heterozygosity has been found in several human neoplasms including breast, hepatic, prostatic, and ovarian cancer (10). Loss of pRb2/p130 expression has been observed in lung cancer (11), lymphomas (12), and choroidal melanoma (13), and RB2/p130 gene mutations have been identified in nasopharyngeal and lung carcinomas (14, 15). We previously reported loss of pRb2/p130 as a frequent event in invasive vulvar carcinomas compared with synchronous premalignant lesions (16). Moreover, we showed that pRb2/p130 is a strong predictor of clinical outcome in endometrial cancer (17).

Ovarian cancer remains a highly lethal disease. In the United States, ovarian malignancy is a leading cause of cancer-related deaths among females and accounts for more deaths than all other gynecological neoplasms combined. Although susceptibility genes such as BRCA1 and BRCA2 have been identified, a majority of ovarian cancers occur sporadically without known risk factors. In addition, most patients present with advanced disease, for which highly effective curative therapy is currently unavailable. It is hoped that a better understanding of the molecular mechanisms underlying the tumorigenic process will lead to earlier diagnosis, novel therapies, and, ultimately, better outcomes.

Previous studies have shown that loss of heterozygosity at the RB/p105 locus is a frequent event in primary ovarian carci-
nomas, (18) and diminished pRb/p105 expression is related to several clinicopathological indicators of aggressiveness in these tumors (19).

As far as pRb2/p130 is concerned, we demonstrated previously that RB2/p130 acts as a tumor suppressor gene in vivo and in vitro in the SKOV3 ovarian carcinoma cell line (20). In the present study, to investigate a possible role for the RB2/p130 gene in the pathogenesis of ovarian cancer, we performed immunohistochemistry in a series of 45 ovarian carcinoma specimens, 4 cell lines, and 4 nonneoplastic ovarian samples. pRb2/p130 expression was detected in benign ovarian surface epithelium but was lost or decreased in 40% of ovarian carcinoma samples. Results from Western blot analysis and reverse transcription-PCR (RT-PCR) were consistent with immunohistochemical data, suggesting a transcriptional regulation of RB2/p130. Consequently, the presence of genomic mutations and promoter hypermethylation was also investigated. Finally, we demonstrated growth arrest of the human ovarian carcinoma cell lines CAOV3 and A2780 using an adenovirus carrying the RB2/p130 gene (Ad-CMV RB2/p130).

These results support a role for RB2/p130 as a tumor suppressor gene in human ovarian cancer and suggest that loss of pRb2/p130 could potentially represent an important marker in the diagnosis and therapy of ovarian carcinoma.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The two ovarian carcinoma cell lines A2780 and CAOV3 were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mM l-glutamine, and the antibiotics streptomycin (10 mg/ml) and penicillin (100 units/ml) in a humidified atmosphere of 5% CO2 at 37°C. OVCAR-4 cells were grown in DMEM supplemented with 10% FCS. OVCAR-8 human ovarian carcinoma cells were maintained in RPMI 1640 with 10% heat-inactivated fetal bovine serum.

Tissue Samples. Ovarian carcinoma tissues were obtained from patients who underwent surgery in the Department of Gynecology of both Thomas Jefferson University (Philadelphia, PA) and the Catholic University of the Sacred Heart (Rome, Italy). Histological classification of tumors was carried out according the WHO system, and tumors were graded as well (G1), moderately (G2), and poorly differentiated (G3). After surgery, each tumor was divided into two portions: one portion was instantly frozen (−80°C) for protein, DNA, and RNA extraction, whereas the second portion was immediately formalin fixed and then paraffin embedded for routine and immunohistochemical investigation.

Immunohistochemistry. Immunohistochemistry was performed as described previously (17). Briefly, ovarian carcinoma tissues were sectioned, fixed, and mounted on slides. Sections were dried in a 50°C oven for 30 min, deparaffinized in xylene, dehydrated using a series of alcohols (100%, 90%, and 85%), and washed in PBS (pH 7.4). Endogenous peroxidase was blocked with 0.5% H2O2. Antigen retrieval treatment was performed in a microwave oven at 300 W for 30 min in 10 mM citrate buffer solution (pH 6). After blocking with normal serum (Lab Vision Corp., Fremont, CA) the slides were incubated with rabbit polyclonal antibody against pRb2/p130 (C-20; sc-317; Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:500 dilution for 1 h. Vectastain ABC Kit and DAB Substrate Kit (Vector, Burlingame, CA) were used to visualize antibody binding. The slides were reviewed by two experienced pathologists (G. F. Z. and C. M.) and scored for the percentage of positive nuclei. In each sample, at least 20 high-power fields were chosen randomly, and approximately 2,000 cells were counted. A number of tumors showed heterogeneous p130 expression. In these cases, the tumor was scored according to the area of highest grade because this was most likely to dictate prognosis. Tumor sections were considered negative if nuclear staining was absent or present in <20% of tumor cells. A cutoff level of 20% was statistically significant and, therefore, functionally operative. Statistical analysis was performed using the χ2 test to analyze the distribution of pRb2/p130-positive cases according to histological grading. Statistical significance was considered at P < 0.05.

Western Blot Analysis. Whole cells and ovarian tissue samples were homogenized in radioimmunoprecipitation assay lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS] with protease inhibitors. An equal amount of 50 µg of protein extract was loaded into 7% SDS-polyacrylamide gels, separated electrophoretically, and then transblotted into a nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked with 5% nonfat dry milk in 1× Tris-buffered saline-Tween and incubated with the primary monoclonal antibody anti-pRb2/p130 (Transduction Laboratories, Lexington, KY) or with the primary polyclonal antibody anti-pRb2/p130 against the COOH terminus of the protein (C-20; sc-317; Santa Cruz Biotechnology) at a dilution of 1:500 in 3% milk overnight at 4°C. After three washes in 1× TBST, the membrane was incubated with the horseradish peroxidase-conjugated secondary antibody (Amersham Life Science, Arlington, IL) diluted 1:10,000 for 1 h at room temperature and detected using enhanced chemiluminescence (Enhanced Chemiluminescence Kit; Du Pont New England Nuclear, Boston, MA).

RT-PCR and Southern Blot Analysis. Total RNA was extracted using Trizol RNA isolation reagent (Molecular Research Center, Cincinnati, OH). Five µg of total RNA were transcribed using the Reverse Transcription System (Promega, Madison, WI). Two µl of cDNA for each sample were used to perform the PCR. The reaction profile consisted of one cycle at 94°C for 3 min; followed by 25 cycles at 94°C for 45 s, 48°C for 1 min, and 72°C for 45 s; and 1 elongation cycle at 72°C for 5 min. The primers used for RB2/p130 were 5′-CCCGGGTTGTTATTGAG-3′ (forward primer) and 5′-AATACCGATAAATCCAT-3′ (reverse primer). The reaction volume was 50 µl in the presence of 2.5 mM MgCl2 and 0.5 µM of each primer. All other reagents were used according to the suggestions of the manufacturer (Fisher Scientific). The amplification of β-actin cDNA demonstrated the use of equal amounts of total cDNA. PCR amplification consisted of 25 cycles for both β-actin and RB2/p130 primers. To obtain a semiquantitative result from RT-PCR, we used the minimum number of cycles required to obtain a clear signal in the linear range.

The Southern blot hybridization was performed on all of the samples analyzed by RT-PCR to confirm the results obtained. Twenty µl of the PCR product (predicted band size, 752 bp) were electrophoresed on a 2% agarose gel and transferred overnight to a nitrocellulose membrane (Hybond N+; Amer-
sham Pharmacia Biotech) positively charged using 0.5 N NaOH. Filter-immobilized DNA was prehybridized for 2 h at 52°C in a solution containing 5× saline-sodium phosphate-EDTA, 5× Denhardt’s solution, 0.5% SDS, and 100 μg/ml fresh denatured sheared salmon sperm DNA and hybridized for 12 h with a 32P-end-labeled oligonucleotide corresponding to the region from nucleotide 1546 to nucleotide 1575 of the sequence (21) using 1 × 10^6 cpm/ml. Probe labeling, hybridization, and washing were performed as described previously (22).

**PCR and Single-Strand Conformational Polymorphism Analysis.** Specific genomic DNA fragments ranging from exons 19 to 22 of the RB2/p130 gene were amplified by PCR and analyzed by single-strand conformational polymorphism as described previously (23).

5-Azacytidine (5-aza-dC) Treatment. 5-aza-dC was dissolved in water at a concentration of 10 mM. OVCAR-4 and OVCAR-8 cells were plated at 3 × 10^5 cells/plate and treated with 5-aza-dC for 6 days, replacing the drug at the fourth day of treatment. The concentration range used was 0.075–10 μM (0.075, 0.1, 1, 5, and 10 μM).

**Adenoviral Production and Transduction.** Ad-CMV-RB2/p130 was generated by subcloning the full-length open reading frame of the RB2/p130 gene into the pAd.CMV-Link1 vector as described previously (24). The pAd.CMV-Link1 vector alone was used as a negative control. CAOV3 and A2780 cells were plated at 5 × 10^5 cells/plate in 10% heat-inactivated fetal bovine serum DMEM and then transduced in 2% heat-inactivated fetal bovine serum DMEM with Ad-CMV-RB2/p130 at a multiplicity of infection of 800 and with Ad-CMV as a negative control. The medium was changed after 24 h of transduction. The cells were harvested after 48 h from transduction and then analyzed by Western blot and fluorescence-activated cell-sorting analysis. The efficiency of transduction was evaluated by transducing the cells with Ad-CMV-GFP at the same multiplicity of infection.

**Fluorescence-Activated Cell-Sorting Analysis.** Cells were analyzed for DNA content as a function of cell number by FACScan (Immunocytometry System; Becton Dickinson, San Jose, CA).

**RESULTS**

**pRb2/p130 Expression in Primary Ovarian Carcinoma.** pRb2/p130 expression was determined by immunohistochemical analysis. Immunoreactivity for pRb2/p130 was found in both nonneoplastic and neoplastic tissues. Strong pRb2/p130 expression was detected in ovarian surface epithelium and stromal cells of benign nonneoplastic ovarian tissue. pRb2/p130 immunostaining was mostly nuclear; however, weak cytoplasmic staining was also observed (Fig. 1A). A total of 45 epithelial ovarian tumors were evaluated. Histopathological diagnoses included 32 serous adenocarcinomas, 7 endometrioid adenocarcinomas, and 6 undifferentiated adenocarcinomas.

Primary ovarian adenocarcinomas showed loss or decrease of pRb2/p130 expression in 18 of 45 cases (40%; Fig. 1D), whereas the presence of the protein was observed in the remaining 60% of cases (Fig. 1, B and C). In contrast to benign nonneoplastic epithelium, where pRb2/p130 was localized mostly to the nuclei, ovarian adenocarcinomas showed cytoplasmic staining as well. Interestingly, we found an inverse correlation between nuclear expression of pRb2/p130 and the nuclear grade (P < 0.05; Table 1). We decided to investigate by Western blot analysis whether similar pRb2/p130 expression was present in the same set of primary ovarian carcinomas and four ovarian carcinoma cell lines (A2780, CAOV3, OVCAR-4, and OVCAR-8). To this end, we used both antibodies against the NH2-terminal and the COOH-terminal region of the protein to exclude the presence of an incomplete gene product. Considering that epithelial ovarian tumors arise in ovarian surface epithelium, the blots included total lysate from immortalized human ovarian surface epithelium cells as an internal control. Samples showing a pRb2/p130 signal at least equal to that of the control cell line were scored as positive. To verify that the same amount of total protein extract was present in each lysate, the same blot was incubated with a monoclonal antibody against β-actin. pRb2/p130 was expressed at different levels, and loss or decrease of the protein was confirmed in 40% of the samples (Fig. 2A). No correlation was found between the different tumor histotypes and the expression of pRb2/p130 evaluated by both immunohistochemistry and Western blot analysis.

**RT-PCR Analysis of RB2/p130 Gene Expression.** We investigated by semiquantitative RT-PCR analysis whether similar RB2/p130 expression was present in a subset of 20 representative ovarian samples and in the 4 cell lines A2780, CAOV3, OVCAR-4, and OVCAR-8. mRNA was subjected to reverse transcription and analyzed by PCR. Fig. 2B shows the varying expression levels of RB2/p130 in the A2780 and CAOV3 cell lines, as well as in five representative samples of ovarian carcinoma, compared with the benign ovarian tissue. Consistent with the immunohistochemical and Western blot data, RB2/p130 expression was detected in benign tissue. The majority of the primary invasive carcinomas (60%) showed RB2/p130 expression, whereas decreased expression was present in eight samples (40%). These results were consistent with the Western blot findings. Fig. 2B shows that RB2/p130-specific transcript was detectable in all but one of the ovarian samples but was considerably weaker compared with benign ovarian surface epithelium in a significant number of ovarian tumors (40%), suggesting a transcriptional repression of the RB2/p130 gene in ovarian cancer. However, lack of correlation between mRNA and pRb2/p130 protein levels in some samples (CAOV3 and sample 4) suggests that posttranscriptional mechanisms, such as proteasome-mediated degradation (25, 26), may also play a role in the regulation of pRb2/p130 expression in ovarian cancer.

As a control, the same reverse transcription products were coamplified with primers targeted to β-actin, and the product was identified in all samples (Fig. 2B).

**Mutational Analysis of the RB2/p130 Gene.** Because RB2/p130 maps on chromosome 16q12.2, in which deletions have been found in several human neoplasms including breast, ovarian, hepatic, and prostatic cancer (10), we analyzed 18 ovarian tissue samples for mutations in exons 19–22, the region that encodes the B domain and the COOH terminus of the protein where the nuclear localization signal is located. As we described previously, mutations in these exons have been detected in several types of tumors (14, 15, 23) and result in either frameshifts after insertions, point substitutions, or abnormal localization of the proteins. All four exons were amplified by PCR and analyzed by single-strand con-
formational polymorphism gel analysis. Then, we directly sequenced the samples to ensure that we had not missed any shifts on single-strand conformational polymorphism. No changes were found on direct sequencing of the exons. Thus, no mutations were found in the ovarian carcinoma cell lines (OVCAR-4 and OVCAR-8) and tumor samples analyzed.

Decreased pRb2/p130 Expression Is Not Related to CpG Island Methylation in Ovarian Carcinoma Cell Lines. Because the RB2/p130 gene promoter consists of several CpG islands, we hypothesized DNA silencing by promoter hypermethylation as a mechanism involved in pRb2/p130 down-regulation. Promoters silenced by methylation can be reactivated by treatment with 5-aza-dC, which is a well-documented inhibitor of DNA methylation. Among the ovarian carcinoma cell lines studied, we selected OVCAR-4 and OVCAR-8, which expressed low levels of RB2/p130 at the mRNA and protein

<table>
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<th>Table 1</th>
<th>Distribution of pRb2/p130 immunostaining according to tumor grade</th>
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<td>Primary ovarian carcinomas ($n = 45$).</td>
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<tr>
<td>pRb2/p130 staining</td>
<td>Grade 1–grade 2</td>
<td>Grade 3</td>
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<tr>
<td>Positive ($\geq 20%$)</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>Negative ($&lt;20%$)</td>
<td>3</td>
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Fig. 1  pRb2/p130 immunostaining in benign nonneoplastic ovarian surface epithelium and primary ovarian adenocarcinomas. A, strong pRb2/p130 expression detected in the nucleus of benign ovarian surface epithelium and stromal cells ($\times 40$, immunohistochemical staining). B, pRb2/p130 expression in an ovarian serous adenocarcinoma with low nuclear grade, where $90\%$ of the neoplastic cells show strong nuclear staining and faint but diffuse cytoplasmic staining ($\times 40$, immunohistochemical staining). C, decreased pRb2/p130 in an ovarian serous adenocarcinoma with intermediate nuclear grade; scattered positive nuclei and diffuse but faint cytoplasmic staining are present in $<10\%$ of cells (image shows a positive field; $\times 40$, immunohistochemical staining). D, faint (virtually absent) cytoplasmic and negative nuclear expression of pRb2/p130 in an ovarian serous adenocarcinoma with high nuclear grade ($\times 40$, immunohistochemical staining).

Fig. 2  A, Western blot analysis of pRb2/p130 expression in benign ovarian surface epithelium (OSE), ovarian carcinoma cell lines (A2780 and CAOV3), and primary ovarian adenocarcinomas (1–5). B, analysis of RB2/p130 expression by reverse transcription-PCR in the same series of samples. Normalization of the samples was carried out with $\beta$-actin.
In developed countries, ovarian cancer accounts for more deaths than all other gynecological malignancies combined. As the result of advances in surgical management and chemotherapeutic options over the last three decades, median survival for ovarian cancer patients has improved. However, overall survival has not been significantly altered. A better understanding of the genetic events involved in the pathogenesis of ovarian carcinoma may help in both the diagnosis and treatment of this malignancy.

pRb2/p130 was the last member of the Rb family to be identified (1). Like pRb/p105 and p107, it has well-characterized cell growth-suppressive properties similar to, yet distinctive from, the other Rb family members (3, 28). In a previous study in nude mice, we showed that ectopic expression of pRb2/p130 suppresses the tumorigenicity of the SKOV3 ovarian carcinoma cell line overexpressing erbB-2 both in vitro and in vivo (20).

In the present study, we examined for the first time the expression of the Rb family member pRb2/p130 in benign nonneoplastic ovarian tissue, ovarian carcinoma cell lines, and a series of primary ovarian carcinomas. Abundant expression of pRb2/p130 was detected in benign tissues compared with primary ovarian tumors. The high percentage of primary ovarian adenocarcinomas exhibiting loss of pRb2/p130 expression is consistent with the aggressive behavior of these tumors, as reported previously for prostate and lung cancer (11, 27).

The immunohistochemical expression of pRb2/p130 was inversely correlated with tumor grade in our series of ovarian carcinomas. This result is consistent with other reports regarding lymphomas (12), lung (11), and breast (29) cancer and further supports our previous data showing an involvement of pRb2/p130 in the differentiation process of several human epithelial tissues (30). pRb2/p130 expression in ovarian carcinoma is frequently cytoplasmic, as observed previously in other malignancies (16, 27). The mechanism responsible for this phenomenon and its biological significance in ovarian carcinoma cells are still unknown. However, it is noteworthy that the nucleocytoplasmic shuttling of E2F4 and the release of its transcriptional activity have been linked to the subcellular compartmentalization of pRb2/p130 (31) and that pRb2/p130 cytoplasmic staining has been associated with a higher aggressiveness of the tumor (27).

Loss of pRb2/p130 in ovarian carcinoma appears to be transcriptionally regulated, as observed previously for endometrial cancer (32). However, lack of correlation between mRNA and pRb2/p130 protein levels in some samples suggests that posttranscriptional mechanisms, such as proteasome-mediated degradation (25, 26), might also play a role in the regulation of pRb2/p130 expression in ovarian carcinoma.

Recent evidence suggests that loss of the pRb2/p130 protein in cancer is attributable to genomic mutations, as observed previously in cell lines such as lymphoid (33) and nasopharyngeal cell lines and in human specimens of primary nasopharyngeal carcinomas (14), lung tumors (15), and Burkitt’s lymphomas (23). In our study, none of the ovarian tumor samples showed the presence of genomic mutations. This finding is in agreement with a recent report on 43 sporadic ovarian tumors (34), suggesting that mutations in the COOH terminus are not responsible for the transcriptional regulation of Rb2/p130.

To evaluate whether epigenetic silencing of RB2/p130 via methylation of the promoter CpG island is involved in ovarian cancer, we treated OVCAR-4 and OVCAR-8 ovarian carcinoma cell lines with 5-aza-dC. RB2/p130 expression was not increased by 5-aza-dC treatment, suggesting that methylation does not have a role in the control of RB2/p130 expression in ovarian cancer. Thus, it is conceivable that other mechanisms, such as promoter regulation, could be involved in the transcriptional regulation of RB2/p130 as shown previously for other Rb family members (35).
Ad-CMV-RB2/p130 enhances pRb2/p130 protein synthesis and leads to a drastic G1 growth arrest in CAOV3 and A2780 ovarian carcinoma cells, thus suggesting a potential tumor suppressor function of pRb2/p130 in this malignancy. Consequently, we hypothesize that a therapy based on the administration of pRb2/p130 could be effective in the inhibition of ovarian carcinoma cell growth.

In summary, the results of our study show that loss or decrease of pRb2/p130 is a frequent event in ovarian carcinoma and that pRb2/p130 expression is inversely correlated with the degree of tumor differentiation. Moreover, the enhanced expression of this protein leads to growth arrest of ovarian carcinoma cell lines, suggesting the putative role of pRb2/p130 as a tumor suppressor in ovarian carcinoma.

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