p21 Expression Predicts Outcome in p53-null Ovarian Carcinoma

Stephen L. Rose, Michael J. Goodheart, Barry R. DeYoung, Brian J. Smith, and Richard E. Buller

The Holden Comprehensive Cancer Center, Division of Gynecologic Oncology, Departments of Obstetrics and Gynecology [S. L. R., M. J. G., R. E. B.], Pathology [B. R. D.], and Biostatistics [B. J. S.], The University of Iowa Hospitals and Clinics, Iowa City, Iowa 52242

ABSTRACT

Purpose: p21 is a direct p53 response gene. Although several studies have correlated p21 and p53 expression, only one has evaluated p21 expression as a function of sequenced p53 gene mutation. We hypothesize that such an analysis may be useful in prognosticating outcome for individuals diagnosed with epithelial ovarian cancer.

Experimental Design: DNA from the primary ovarian cancers of 267 patients was studied. p53 mutations were directly sequenced. Two percent or greater nuclear staining with WAF1/CIP1 monoclonal antibody was determined by a hazard ratio analysis to constitute positive p21 expression.

Results: Positive p21 nuclear staining occurred more frequently in p53 wild-type ovarian tumors than tumors found to have a p53 mutation (P = 0.001). Positive p21 staining conferred an overall survival advantage (P = 0.02). p21 expression in cancers with p53 missense mutations was not prognostic but did show a strong trend toward significance in the wild-type p53 subset (P = 0.056). Surprisingly, positive p21 staining reflected compromised survival for individuals with p53-null ovarian cancers (P = 0.005). The mean expression level for p21-positive stains in the wild-type group was greater than in null p53 cancers (23 versus 11%; P = 0.001). A Cox multivariable analysis revealed p21 to be a strong independent prognostic factor in p53-null ovarian cancer (P = 0.02).

Conclusion: p21 expression is closely related to sequenced p53 mutations. This is the first study of positive p21 staining as an independent poor prognostic factor in p53-null ovarian cancer. A dual role for p21 activity, dependent on levels of expression, appears to explain these paradoxical results and is consistent with a complex model for regulation of p21.

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2 To whom requests for reprints should be addressed, at 200 Hawkins Drive, 4630 JCP, Iowa City, IA 52242. Phone: (319) 356-2015; Fax: (319) 353-8363; E-mail: richard-buller@uiowa.edu.

INTRODUCTION

Ovarian carcinoma is the deadliest gynecologic malignancy. There will be an estimated 23,300 new cases and 13,900 deaths in the United States alone in 2002 (1). The mortality rate remains high because of the delay in diagnosis that characterizes ovarian cancer. Because of this poor prognosis, many attempts have been made to study newer prognostic indicators that may prove to be useful on an individual patient basis.

p53 is a tumor suppressor gene that encodes a M, 53,000 nuclear phosphoprotein involved in Gap 1 (G1) cell cycle arrest (2). p53 dysfunction is one of the most common genetic alterations found in cancer, occurring in ≈50% of all human malignancies (3). We have shown previously that p53 is mutated in ~60% of ovarian malignancies and that p53-null mutations are independent molecular predictors of compromised survival (4).

p21 is a direct p53 response gene but has also been shown to be inducible by p53-independent pathways (2, 5–10). It is a cdk inhibitor that directly inhibits cdk 4 and 6 from complexing with cyclin D. This inhibition leads to sequestration of transcription factor E2F and arrest of cellular proliferation at the restriction point in G1 (2). p21 expression as measured by immunohistochemical staining has been shown to be positively associated with survival in gastric, anal, prostate, and breast cancers (11–15). Although some studies have shown a survival benefit to increased p21 expression in ovarian cancer, the role of p21 as an independent prognostic indicator is still controversial (16–24).

Many researchers have evaluated the relationship of p21 expression to p53 status by p53 immunostaining. Our group has shown in previous studies that p53 immunostaining cannot detect 33% of sequenced p53 mutations, whereas it is falsely positive in 23% of tumors with wild-type p53 sequence (4, 25). For the present study, we sequenced the p53 gene or exons suggested to contain p53 mutation based on SSCP screening, to control for actual p53 mutation status, and investigate the survival relationship between p21 expression and p53 gene mutation. The hypothesis tested was that p21 expression serves as a better measure of p53 function than a simple p53 abnormality implied by immunostaining or sequencing.

PATIENTS AND METHODS

Study Population. A total of 267 patients were seen either initially or in consultation for the diagnosis of primary, invasive epithelial ovarian cancer at The Holden Comprehensive Cancer Center of The University of Iowa. Diagnosis was between January 1, 1990 and December 31, 1998. The study was carried out in accordance with the standards of the Institutional Human Subjects Protection Review Board.

2 The abbreviations used are: cdk, cyclin-dependent kinase; SSCP, single-strand conformation polymorphism; FIGO, International Federation of Gynecology and Obstetrics; LSAB, labeled streptavidin biotin.

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Tissue Preparation. Tumor was obtained from either fresh, snap-frozen specimens (n = 133) or paraffin-embedded samples (n = 134). DNA or cDNA isolation and p53 gene sequencing techniques have been well described in our previous publications (4, 25). p53 mutations were characterized as null (frameshift or nonsense) and missense. We did not attempt to distinguish between expressed wild-type p53 sequence and potentially unexpressed sequences.

p53 Analysis. Because of the infrequency of p53 gene mutations in exons 2, 3, and 11 reported previously, only exons 4–10 were studied (4, 26). Exons 4–10 were directly sequenced using laser identification of fluorescent labeled dideoxy chain terminating nucleotides. We were unable to completely sequence some samples because of poor PCR amplification of DNA extracted from paraffin-embedded tissue. In these cases, SSCP gels were performed. All together, 49% of the samples had SSCP gels performed at one or more exons. Most of the products could, however, be screened on SSCP gels. In the case of an abnormal SSCP gel, DNA was re-extracted, and the exon in question was sequenced to confirm the presence or absence of mutation.

p21 Staining. The WAF1/CIP1 monoclonal antibody to p21 was used for immunohistochemical staining of the tumor sections. An immunoperoxidase reaction for p21 was performed on each case using LSAB Plus. Sections were deparaffinized in xylene, rehydrated in graded alcohols, and rinsed. Antigen unmasking was accomplished using citrate buffer at a pH of 6 in the microwave for 2.5-min cycles at high power. Slides were allowed to cool for 20 min at room temperature. Endogenous peroxidase activity was quenched using hydrogen peroxide in distilled water. Sections were then covered with p21 primary antisera, used at a dilution of 1:10, incubated for 1 h at room temperature, and rinsed. Sections were then covered with LSAB Plus Linking Antibody for 30 min at room temperature, rinsed, and then covered with LSAB Plus streptavidin label for 30 min at room temperature and rinsed again. Sections were incubated with diaminobenzidine to demonstrate the signal of the primary antibody. A counterstain of Mayer’s Hematoxylin was used for 1 min. Negative control slides were prepared by substituting irrelevant mouse IgG for WAF1/CIP1 antibody. p21 antibody and detection agents were purchased from DAKO, Corp. (Carpinteria, CA). All rinses were performed using PBS/brij. A single pathologist (B. R. D.), blinded to tumor p53 status, reviewed the stains. p21 staining was reported as negative in the absence of any nuclear staining. A positive stain was reported as a percentage of sample nuclei taking up stain. p21 staining was quite variable both between and within tumors. We performed exploratory analyses to maximize the hazard ratio for a “positive” p21 stain and its impact on survival as determined by the Log-rank test. Two percent nuclear staining accomplished this goal. Therefore, all tumors with <2% nuclear staining were deemed p21 negative.

Statistical Analysis. Frequency tables and descriptive statistics are presented to summarize the data. The Pearson χ² test was used to measure the association between p21 status and the remaining predictor variables. Log-rank tests were carried out to compare the median survival times across p21 status. Kaplan-Meier plots were constructed to provide estimates of the survival functions. The multivariable effect on survival was modeled using Cox proportional hazards regression. Analyses were performed with the SAS statistical software package, version 8 (SAS Institute, Inc., Cary, NC, 2001).

RESULTS

Among the 267 patients entered, mean age at diagnosis was 57 years. Patient follow-up was last surveyed in February 2002; at that time, median follow-up was 3.9 years (range: 0.02–11.7 years). Minimum follow-up of surviving patients was 3.5 years. The pathologic characteristics and p21 staining patterns of the study cancers are listed in Table 1.

The overall p53 mutation rate was 48%. p53 mutations were more common in advanced stage cancers (stage III and IV) than in earlier stage cancers (stages I and II; P < 0.001). Positive p21 stains (≥2% nuclear staining; see “Materials and Methods”) were found in 123 (46%) of the tumor samples. As found in previous studies, positive p21 staining was recorded more often for lower stage tumors (P = 0.003), tumors of clear cell histology (P = 0.001), and those optimally cytoreduced (P = 0.02; Refs. 16, 20, 21, and 27). Wild-type p53 tumors displayed a higher proportion of positive p21 staining than did tumors with either missense or null p53 mutations (P = 0.04). Of the positive stains within these groupings, mean p21 expression was as follows: (a) wild-type p53, 23%; (b) missense p53, 16%; and (c) null p53, 11%. The difference between p21 expression level in wild-type p53 tumors and p53-null tumors was

Table 1 Patient characteristics and P21 staining

<table>
<thead>
<tr>
<th>Overall</th>
<th>Stage</th>
<th>Positive p21 stain (%)</th>
<th>P</th>
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<tbody>
<tr>
<td>Overall 267 123 (46)</td>
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<td></td>
<td></td>
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<tr>
<td>Stage</td>
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<tr>
<td>I 68 (26) 42 (62)</td>
<td>0.003</td>
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<tr>
<td>II 17 (6) 11 (65)</td>
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<tr>
<td>III 139 (52) 56 (40)</td>
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<tr>
<td>IV 43 (16) 14 (33)</td>
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</tr>
<tr>
<td>Tumor grade</td>
<td></td>
<td></td>
<td>0.36</td>
</tr>
<tr>
<td>Well 33 (12) 19 (58)</td>
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<td></td>
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</tr>
<tr>
<td>Moderate 80 (30) 36 (45)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Poor 154 (58) 68 (44)</td>
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<tr>
<td>Tumor histology</td>
<td></td>
<td></td>
<td>0.001</td>
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<tr>
<td>Papillary serous 136 (51) 49 (36)</td>
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<tr>
<td>Endometrioid 55 (21) 35 (64)</td>
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<tr>
<td>Mucinous 40 (15) 18 (45)</td>
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<tr>
<td>Adenocarcinoma NOS* 14 (5) 5 (36)</td>
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<tr>
<td>Clear cell 19 (7) 15 (79)</td>
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<tr>
<td>Transitional cell 3 (1) 1 (33)</td>
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<tr>
<td>P53 status</td>
<td></td>
<td></td>
<td>0.04</td>
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<tr>
<td>Wild type 139 (52) 72 (52)</td>
<td></td>
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<tr>
<td>Missense 85 (32) 38 (45)</td>
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<tr>
<td>Null 43 (16) 13 (30)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Residual disease</td>
<td></td>
<td></td>
<td>0.02</td>
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<tr>
<td>0–1.0 187 (70) 95 (51)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>&gt;1.0 80 (30) 28 (35)</td>
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* Not otherwise specified.
significant \( (P = 0.04) \). There was no relationship between positive p21 staining and tumor grade.

Univariate survival analysis for the entire cohort revealed that p21 positive was associated with a survival benefit (Fig. 1a). Median survival was 6 years for p21-positive tumors and 4 years for p21-negative tumors \( (P = 0.02) \). When only p53 wild-type tumors were evaluated, there was a trend toward a survival advantage in p21-positive cancers with median survival of 9 years in the p21-positive group and 4.1 years in the p21-negative group \( (P = 0.056) \). The median survival for the p53 missense cancers with p21-positive staining \( (4.8 \text{ years}) \) was not significantly different from the p21-negative staining tumors \( (4 \text{ years}; P = 0.14) \). For p53-null cancers, however, a survival advantage was detected for individuals whose tumors stained negative for p21: median survival 2.4 versus 1.5 years \( (P = 0.005; \text{Fig. 1b}) \). Of the 13 p21-positive staining tumors with p53-null mutation, there was no quantitative correlation between percentage of nuclei staining positive and survival, although this varied from 2 to 40%.

Next, a Cox multivariable regression analysis was carried out, including all prognostic factors in the data set. Age at diagnosis, FIGO stage, and residual disease were independent prognostic factors of survival in ovarian cancer for the group as a whole (Table 2). FIGO stage was significantly independent of p53 mutation status. Age \( (P = 0.003) \), grade \( (P = 0.045) \), and residual disease \( (P < 0.001) \) were only significant for p53 wild-type cancers. p21 staining was a significant independent outcome variable for p53-null tumors \( (P = 0.02) \).

**DISCUSSION**

p21 has been reported as a useful molecular prognostic factor in many human tumors, including: \( \text{(a) gastric; (b) anal; (c) prostate; and (d) breast cancers (11–15). Studies of p21 as a prognostic factor in ovarian cancer have produced mixed results (16–22). p21 expression is expected to be associated with enhanced survival, but this has only achieved significance in a multivariate model for p53-negative staining cancers (20, 23). Other studies failed to detect a role for p21 expression as a factor modifying survival even in a univariate model (18, 19, 22). Perhaps this is because there is no uniform definition of what constitutes a positive p21 stain. A wide variety of thresholds for a positive stain have been evaluated. These range from a low of any nuclear staining to as high as 75% for a strong positive stain (16, 18–22). We have defined a positive stain as \( <2\% \) nuclear staining based on a statistical evaluation of our entire data set to maximize the hazard ratio. However, this approach is not without criticism (28).

In the present study, we also see a trend to survival benefit for wild-type p53/p21 positive tumors, but that does not quite reach significance \( (P = 0.056) \). We were surprised to find compromised survival for patients with p53-null/p21-positive tumors. To our knowledge, this is the first such group of patients to show that p21 alone is an independent prognostic factor when analyzed in a multivariable analysis.

This is the only sizable study of any cancer to relate p21 expression to sequenced p53 mutations. Sequenced wild-type p53 cancers do indeed express higher p21 levels than do cancers with a mutant p53 gene sequence. We also confirm a significant increase of positive p21 stains in tumors of clear cell histology as compared with other histological subtypes, as shown in previous reports (20, 27). Just as low-stage and clear cell ovarian carcinomas are less likely to harbor p53 gene mutation than

![Fig. 1](https://clincancerres.aacrjournals.org)
high-stage and serous carcinomas, p21 expression mirrors the p53 mutation status of these cancers.

The combination of p21 expression data with p53 mutation status, considered along with conventional parameters known to modify ovarian cancer survival, afforded us the opportunity to make some unique observations. As might be expected, overall, individuals with p21-expressing tumors have a better outcome than those with p21-negative cancers. The same observation held true for cancers containing wild-type p53 gene sequences. However, our multivariable analysis suggests that this is probably attributable to less residual disease and lower FIGO stage associated with the p21-positive cancers. We were hoping to use p21 expression as a marker for functional discrimination between ovarian cancers with a wide variety of p53 missense mutations based on the premise that not all missense mutations are functionally equivalent (29). This goal was not achieved either in a univariate or multivariable model. The analysis of p53-null cancers, a previously identified poor prognostic group (4, 30), provided a unique result. Not only was p21 expression significant in the univariate model, but it also emerged as the most significant prognostic factor in a multivariable model. The paradoxical observation that lower p21 expression was a favorable molecular prognostic factor forces us to look more closely at the regulation of p21 and the role it may play in the cell cycle.

Although the finding of compromised survival among p53-null/p21-positive tumors is counterintuitive, it is consistent with the observations of LaBaer et al. (5). These investigators used human tumor cell lines to study the effects of various cdk inhibitors on cdk/cyclin formation. They found that p21 displays two separate, but overlapping, effects on cdk4/cyclin D formation. At lower concentrations, cdk4/cyclin D formation was actually promoted, whereas at higher p21 concentrations, complex formation was inhibited (5). This dual role for p21 had been suggested earlier by Zhang et al. (31), who demonstrated that p21 was permissive of cdk2 activity at low levels but was inhibitory at higher, saturating levels. Of the p21-positive, p53-null tumors identified in our group, none demonstrated >40% nuclear staining. In fact, the mean p21 expression in this group was only 11% compared with 23% for the p21-positive, wild-type p53 cancers. Thus, we postulate that the unfavorable input of p21 on survival is attributable to an increase in cdk4 activity promoted by lower p21 levels from p53-independent pathways for the former group, whereas p53-dependent pathways upregulate p21 expression in the latter group. This dual threshold may in fact also contribute to the lack of consistency between reports that have not included p53 gene sequencing as a measure of p53 status.

In conclusion, we are the first to report p21 expression as an independent prognostic factor in p53-null ovarian cancers. We would suggest that this paradoxical survival benefit confirms in vivo what previous in vitro studies have suggested based on cdk4 activation at low p21 levels, followed by inhibition at higher levels of expression (5, 31). Our findings further provide evidence of the complexities of cell cycle regulation. Future studies looking at other modifiers of the cell cycle, such as p14ARF, p16INK4a, and mdm2, will be invaluable in solving the molecular puzzle of cellular proliferation.

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