The p16-Cyclin D1/CDK4-pRb Pathway and Clinical Outcome in Epithelial Ovarian Cancer

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ABSTRACT

A significant positive association has been reported between p16 expression and clinical outcome for epithelial ovarian cancer patients. However, there is a reciprocal correlation between genetic alterations of single members of the p16-cyclin D1/CDK4-pRb pathway (G1 pathway). Simultaneous evaluation of these four elements may produce a better prognostic factor than p16 alone. We studied the prognostic significance of the G1 pathway in 59 epithelial ovarian cancer patients undergoing surgery and platinum-based chemotherapy by immunohistochemical technique. Abnormal expression of p16 or pRb was defined by negative nuclei staining, and that of cyclin D4 and cyclin D1 was defined by 50% nuclear staining. An abnormal G1 pathway was indicated in cases that have at least one abnormality among these four elements. Abnormal expression of p16, pRb, and cyclin D1/CDK4 was observed in 33.9, 3.4, and 15.3% of studied cases, respectively. Abnormal G1 pathway was detected in 49.2% (29 of 59) of all cases. The patients with normal G1 pathway tended to achieve a higher complete response rate (81.0%) to chemotherapy, compared with patients with abnormal G1 pathway (55.0%); however, there was no significant difference (P = 0.1001) between the two groups. Univariate analyses identified advanced stage [hazard ratio (HR), 3.665; P = 0.0218], histological low grade (HR, 3.625; P = 0.0066), and abnormal G1 pathway (HR, 2.935; P = 0.03) as prognostic factors for overall survival. The G1 pathway might help as a prognostic factor to select high-risk patients.

INTRODUCTION

Ovarian cancer is the most common cancer in women to be diagnosed at an advanced stage and is the leading cause of death from gynecological malignancy. Fifty % of patients, after optimal surgical debulking and a pathologically complete response to primary chemotherapy, will develop a recurrence of the tumor and will die within 2 years. The development of additional prognostic factors, closely related to tumor cell biology, is essential for identification of patients with a particularly poor prognosis. Recent genetic and biochemical investigations of the molecular mechanisms governing the G1 to S progression in mammalian cells have demonstrated an important role for D-type cyclins and their partner kinases CDK4 and CDK6 (1–4). When activated by cyclin D1, CDK4 is able to phosphorylate pRb, leading to the release of associated proteins like E2F that have the capability to activate genes necessary for cell progression through the G1 phase (4). p16 controls cell cycle proliferation during G1 by inhibiting the ability of cyclin D/CDK4 and cyclin D/CDK6 complexes to phosphorylate pRb (5). The components of the p16-cyclin D/CDK-pRb pathway (G1 pathway) are frequently found to be altered in various types of cancers (6–11). In ovarian cancer, the loss of p16 expression was reported to be detected in 11–37% of studied cases (12–15). It has been reported that overexpression of p16 might be an important early event in ovarian cancer (16) and that high expression of p16 is associated with poor prognosis in ovarian cancer patients (13, 14). However, findings of loss of p16 expression are generally thought to be abnormal. Heyman et al. (17) reported a statistically significant correlation between inactivation of p15/p16 by homozygous deletion or intragenic mutation and poor prognosis. These findings seem contradictory.

In solid tumors, there is a reciprocal correlation between genetic alterations of single members of the p16-cyclin D/CDK4-pRb pathway (18–20). It has been reported that hypophosphorylated active pRb can repress p16 expression, whereas inactivation of pRb by phosphorylation leads to p16 expression (21). Fang et al. (22) reported that expression of p16 induced transcriptional down-regulation of the Rb gene. Consistent with these findings, CDKN2 gene deletion and Rb deficiency are reported to be inversely correlated in many types of tumors (8, 18). In addition, a strong association between altered cyclin D1 and pRb expression has been reported in esophageal tumors (23). Muller et al. (24) have demonstrated that the cell cycle-dependent expression of cyclin D1 in tumor cell lines requires the presence of a functional pRb. Masciullo et al. (25) reported a direct relationship between the expression levels of

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3 The abbreviations used are: CDK, cyclin-dependent kinase; Rb, retinoblastoma; pRb, Rb protein; IHC, immunohistochemistry; CR, complete response; OS, overall survival.
cyclin D1 and CDK4. The expression levels and activities of these proteins can modulate each other. We believe it is important to evaluate these four elements simultaneously. Additionally, to our knowledge, there are no reports in which p16, cyclin D1, CDK4, and pRb were examined simultaneously in epithelial ovarian cancer. In this study, we investigated the prognostic importance of all these key components of this G1 checkpoint by IHC approaches in epithelial ovarian cancer.

MATERIALS AND METHODS

Clinical Samples. All of the specimens analyzed in this study were obtained from patients who were newly diagnosed from 1994 to 1998 at Osaka City General Hospital, Osaka, Japan. Paraffin-embedded tissue of 59 ovarian cancers (stage 1, 19; stage 2, 6; stage 3, 29; and stage 4, 5) were collected. Of the 59 tumors, histology types included 25 serous, 6 mucinous, 11 clear cell, 15 endometrioid, and 2 undifferentiated. All surgical staging and debulking procedures were performed by experienced gynecologists using standard techniques. Histological diagnosis was confirmed by microscopic examination of the H&E-stained sections according to WHO criteria. Clinical stages were determined according to the International Federation of Gynecology and Obstetrics system. All patients with stages 1c, 2, 3, and 4 cancer received postoperative chemotherapy with a platinum-based regimen. Postoperative chemotherapy from 1994 to 1997 was comprised of nine cycles of CAP (cyclophosphamide-Adriamycin-platinum) therapy composed of 500 mg/m² cyclophosphamide, 50 mg/m² doxorubicin, and 50 mg/m² cisplatin. The regimen from 1998 was TP (Taxol-platinum) therapy composed of 180 mg/m² paclitaxel over 3 h, followed by 75 mg/m² cisplatin or AUC5 carboplatin. CR was defined by normalization of the 35 units/ml CA125, resolution of computed tomographic scan abnormalities, and a complete clinical remission of disease. Patients with stage 1 disease were treated with a single surgical debulking procedure. Postoperative chemotherapy was administered for at least 12 cycles.

IHC of p16, pRb, CDK4, and Cyclin D1 Proteins. Histological sections were affixed to glass slides, dewaxed, and rehydrated. Autoclave unmasking process (10 min at 121°C in 10 mM citrate buffer, pH 6.0) was used. The sections were then incubated in 3% hydrogen peroxide for 10 min at room temperature to quench endogenous peroxidase activity. The sections were reacted with one of the following primary antibodies at 4°C overnight: (a) rabbit anti-p16 polyclonal antibody (PharMingen, San Diego, CA); (b) mouse anti-pRb monoclonal antibody (PharMingen, San Diego, CA); (c) rabbit anti-CDK4 monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); (d) mouse anti-cyclin D1 monoclonal antibody (Medical and Biological Laboratories Co., Nagoya, Japan), or nonimmunized rabbit serum. After rinsing, the sections were incubated for 30 min with mouse or rabbit EnVision + Peroxidase (Dako, CA). The peroxidase activity for p16, pRb, CDK4, and cyclin D1 was visualized by applying diaminobenzidine chromogen containing 0.05% hydrogen peroxide for 2–10 min at room temperature. The sections were then counterstained with hematoxylin. Positive and negative control experiments were performed for each tumor staining.

Interpretation of IHC Staining. The slides were read by two professional pathologists who were blinded to the clinical outcome of the patients. Tumors were scored as p16-negative if all malignant cells had no nuclear staining and surrounding normal stromal cells showed adequate nuclear staining as a positive internal control. Tumors were regarded as p16-positive if any malignant cells had nuclear staining. Small lymphocytes, which showed no nuclear staining of p16, were used as a negative internal control (26). The same criteria were used for the evaluation of pRb staining. Negative nuclear staining was considered as abnormal expression of p16 and pRb. Evaluation of CDK4 and cyclin D1 positives was performed by semiquantitative analyses. The following scale was used (27): −, no immunoreactive tumor cells detectable or <5% of the tumor cells were positive with a weak intensity; 1+, 5–50% of the tumor cells were positive with a strong intensity; and 2+, >50% of the tumor cells were positive with a strong intensity. Strong nuclear staining in G1 was considered as abnormal expression of CDK4 and cyclin D1. Abnormal G1 pathway was indicated in cases that have at least one abnormality among these four elements.

Statistical Analysis. Univariate differences between categorical variables were evaluated by using Fisher's exact test. OS distribution was calculated by using the Kaplan-Meier method, and univariate Cox regression analyses were used to identify...
variables associated with OS. All $P$s are for two-sided significance tests.

RESULTS

p16, pRb, CDK4, and Cyclin D1 Expression in Primary Tumor Samples. Associations of abnormal expression of p16, CDK4, cyclin D1, and pRb and clinicopathological features are shown in Table 1. Loss of p16 and pRb and strong expression of CDK4 and cyclin D1 were 33.9, 3.4, 15.3, and 5.1%, respectively. Abnormal G1 pathway was detected in 49.2% (29 of 59) of all cases. Abnormal expression of these four factors was not significantly associated with clinical stage, histological type, or histological grade. Twenty-six of 29 patients (89.7%) with abnormal pathway had only one abnormal factor. There tended to be a reciprocal correlation between genetic alterations of single members of the p16-cyclin D1/CDK4-pRb pathway. Nine of 39 (23.1%) patients with p16 expression had abnormal expression of pRb, cyclin D1 or CDK4, and four of these nine cases (abnormal pathway with p16 expression) died within 3 years. If p16 expression is divided into two groups (strong expression, $\geq 50\%$ of the tumor cells were positive; weak expression, <50% of the tumor cells were positive), four of nine cases (44.4%) with abnormal expression of cyclin D1/CDK4 had strong p16 expression, and only one of 30 cases (3.3%) with

Fig. 1 Typical images of p16, pRb, CDK4, and cyclin D1 staining. A, positive p16 immunostaining. B, negative p16 immunostaining. The section shows negative nuclear staining of the tumor cells. C, positive pRb immunostaining. D, negative pRb immunostaining. Note that stromal cells show distinct nuclear staining (arrowheads) of p16 and pRb, which provide positive internal controls for both proteins. E, strong positive CDK4 immunostaining. F, strong positive cyclin D1 immunostaining. α–f, ×200.
normal pathway had strong p16 expression. All patients were divided into two groups, abnormal G1 pathway and normal G1 pathway. There were no significant differences in mean age (54.4 ± 11.9 versus 54.4 ± 14.7; t test, P = 0.9892). Fisher’s exact tests indicated that the status of the G1 pathway was not associated with clinical stage (P = 0.4348), histological type (P = 0.7948), histological grade (P = 0.7710), and residual disease status (P = 0.7710). Typical images of p16, pRb, CDK4, and cyclin D1 staining are shown in Fig. 1.

**Relationship between the Status of the G1 Pathway and the Clinical Responses to Platinum-based Chemotherapy as First-Line Chemotherapy.** Forty-one of 59 patients were assessable for CR on the basis of persistent elevation of the CA125 level or measurable disease on computed tomographic scan postoperatively. There were no significant differences in mean age, clinical stage, histological type, histological grade, and residual disease status between the abnormal G1 pathway and normal G1 pathway groups. The CR rate to chemotherapy in the cases with assessable disease postoperatively was 68.3% (28 of 41 patients with assessable disease postoperatively). Eighty-one % of patients with normal G1 pathway achieved CR, compared with 55.0% of patients with abnormal G1 pathway. The patients with normal G1 pathway tended to achieve higher CR rate, compared with the patients with abnormal G1 pathway; however, the difference between the two groups was not significant (P = 0.1001).

**Relationship between the Status of the G1 Pathway and OS Rate.** For the entire cohort, the OS was 67.8% (median follow-up, 22 months). As shown in Fig. 2a, OS in loss of p16 was 55.0% (median follow-up, 20 months) and OS in expression of p16 was 74.4% (median follow-up, 27 months), which does not associated with clinical stage (P = 0.4348), histological type (P = 0.7948), histological grade (P = 0.7710), and residual disease status (P = 0.7710). Typical images of p16, pRb, CDK4, and cyclin D1 staining are shown in Fig. 1.

**Table 2** Statistical analysis of the effect of various prognostic factors on OS

<table>
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<th>Variable</th>
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<th>95% CI</th>
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<tr>
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<td>G1 pathway</td>
<td>0.0300</td>
<td>2.935</td>
<td>1.110–7.762</td>
</tr>
</tbody>
</table>

Table 2. Statistical analysis of the effect of various prognostic factors on OS

- Age, ≥60 versus <60 years; Stage, III/IV versus I/II; Histology, nonserous versus serous; Grade, III versus I/II; p16, negative versus positive; CDK4, ≥50% versus <50% cells positive; cyclin D1, ≥50% versus <50% cells positive; pRb, negative versus positive; G1 pathway, abnormal versus normal.
- Hazard ratio refers to risk of death, with values <1.0 indicating reduced risk.
- CI, confidence interval.
not constitute a significant difference \((P = 0.1245)\). The status of pRb, CDK4, or cyclin D1 was not also associated with OS (data not shown). However, OS of 80.0% (median follow-up, 27.0 months) for patients in the normal G1 pathway group was significantly superior to the OS of 55.1% (median follow-up, 19 months) for patients in the abnormal G1 pathway group \((P = 0.0213; \text{Fig. } 2b)\). By univariate analysis, abnormal G1 pathway, advanced stage, and histological grade were significant predictors of poor OS (Table 2).

**DISCUSSION**

It has been reported that loss of expression of p16 in human ovarian tumors was 11–37\% \((12–15)\). Some reports demonstrated that loss of p16 expression was detected mainly in mucinous and endometrioid types and advanced stage cancers \((13–15, 28)\). In this study, loss of p16 expression was 33.9\%, and there was no significant relationship between p16 status and clinical stage, histological grade, or histology. We did not examine the CDKN2 gene status; however, CDKN2 gene mutations are thought to be rare events in ovarian cancer \((12, 14, 28–30)\). Concerning pRb, most ovarian epithelial tumors \((86–96\%) \) show high pRb expression with IHC \((31–33)\). In our series, loss of pRb was detected only in 3.3\% \((3 of 99)\). There are few reports about the abnormalities of CDK4 and cyclin D1 in ovarian cancer \((10, 25)\). In these reports, overexpression of CDK4 and cyclinD1 were 14% \((7 of 48)\) and 18% \((12 of 65)\), respectively, by Northern blot analysis, and these did not appear to be associated with clinical outcome. In our study, strong expressions of CDK4 and cyclin D1 were 15.3 and 5.1\%, respectively, by IHC, and in the strong expression group, 40\% \((4 of 10)\) died within 3 years because of tumor progression. Most cases \((89.7\%: 26 of 29)\) with abnormal G1 pathway had one abnormal factor of p16, CDK4, cyclin D1, or pRb. The abnormalities of p16 and cyclin D1/CDK4 tended to be reciprocal.

To our knowledge, there have been no reports in which the correlation between the response to chemotherapy and G1 pathway was examined in ovarian cancer. In our studies, patients with normal G1 pathway tended to achieve a higher CR rate with a platinum-based regimen, compared with patients with abnormal G1 pathway. Effects of alterations in cell cycle genes on cytotoxicity of chemotherapeutic agents remain unclear. It was reported that p16-mediated G1 arrest prevented platinum-induced cell death \((34, 35)\). Hochhauser et al. \((36)\) demonstrated that there was an increased fraction of cells in the S and G2 phases of the cell cycle among cells expressing higher levels of cyclin D1. Cytotoxicity assays revealed a statistically significant increase in resistance to methotrexate in cells expressing high levels of cyclin D1; however, there was no difference in resistance to doxorubicin and paclitaxel. Fukuoka et al. \((37)\) reported that p16INK4 expression was closely associated with the increased sensitivity of an ectopic p16INK4-expressing non-small cell lung cancer cell line with p16 homozygous deletion to topoisomerase I inhibitors. Effects of alterations in cell cycle genes on cytotoxicity may depend on the type of drug. We performed platinum-based combination chemotherapy including cyclophosphamides and doxorubicin or paclitaxel. Further examination will be required to resolve this problem.

In this study, G1 pathway status proved to be a significant predictor of OS, despite the short follow-up period. In addition, all four patients with stage 1 or 2 who died had abnormal G1 pathways. However, p16, CDK4, cyclin D1, or pRb status alone was not associated with clinical outcome. The G1 pathway was found to be a better prognostic predictor of ovarian cancer than p16, CDK4, cyclin D1, or pRb alone. High expression of p16 was reported to be associated with poor prognosis in ovarian cancer patients \((13, 14)\). These findings seem to contradict our results. We have the following speculations. In this study, the abnormalities of p16 and cyclin D1/CDK4 tended to be reciprocal. Nine of 39 \((23.1\%)\) patients with p16 expression had abnormal expression of pRb, cyclin D1 or CDK4, and four of these nine cases \((abnormal G1 pathway with p16 expression)\) died within 3 years. In our study, four of nine cases \((44.4\%)\) with abnormal expression of cyclin D1/CDK4 had strong p16 expression; however, only 1 of 30 cases \((3.3\%)\) with normal G1 pathway had strong p16 expression. Strong p16 expression tended to be associated with abnormal expression of cyclin D1/CDK4. Yao et al. \((38)\) reported that CDK4 overexpression accompanied elevated p16 status and that elevated p16 levels might be the result of compensatory up-regulation of this protein to counteract CDK4 overexpression in primary soft-tissue sarcoma. It may be that some of the cases with high p16 expression in former reports \((13, 14)\) also had abnormal expression of pRb, cyclin D1, or CDK4. Status of the G1 pathway was associated with clinical outcome in ovarian cancer patients. However, p16, CDK4, cyclin D1, or pRb status was not a good clinical predictor because some patients with expression of p16 also exhibited abnormal expression of pRb, cyclin D1, or CDK4. In conclusion, the G1 pathway is a useful prognostic predictor in ovarian cancer.

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