

Cyclin E – Associated Kinase Activity Predicts Response to Platinum-Based Chemotherapy

Isabelle Bedrosian,¹ Christine Lee,² Susan L. Tucker,³ Shana L. Palla,³ Karen Lu,² and Khandan Keyomarsi⁴

Abstract Purpose: The role of cyclin E as a predictive marker of response to chemotherapy remains unknown. We have previously shown that deregulation of cyclin E in an ovarian tumor cell line model enhances cyclin E – associated kinase activity and sensitizes tumor cells to cisplatin. We hypothesized that cyclin E deregulation would predict for responsiveness to platinum-based regimens in ovarian cancer patients.

Experimental Design: Patients who met the following criteria were retrospectively identified from the institutional tumor bank records: (a) high-grade ovarian epithelial malignancy, (b) stage III/stage IV disease, (c) optimally debulked, (d) completed platinum-based therapy. Tumor samples were analyzed for cyclin E, p21, and p27 by Western blot analysis and assessed for cyclin E – associated kinase activity.

Results: Seventy-five patients, who met the study criteria, were identified. Cyclin E protein levels did not correlate with cyclin E – cdk2 kinase activity (Spearman's rho, 0.07; $P = 0.58$). Cyclin E – associated kinase activity was the only significant predictive marker for response to platinum-based therapy, with higher response rates seen in patients with higher levels of activity ($P = 0.045$). Cyclin E protein levels did not predict for platinum sensitivity ($P = 0.20$). In contrast, cyclin E protein levels, but not cyclin E – associated kinase activity, was a significant predictor for freedom from recurrence ($P = 0.01$ and $P = 0.25$, respectively).

Conclusions: Cyclin E overexpression and cyclin E – associated kinase activity have distinct roles in predicting for response to chemotherapy and outcome in ovarian cancer patients. These results suggest a compartmentalization of cyclin E functions in the oncogenic process.

Overexpression of the cyclin E protein has been linked to shortening of the G₁ phase of the cell cycle (1), decreased requirement for growth factors (1), enhanced cell proliferation (2), induction of chromosomal instability (3, 4), and polyploidy (5). These processes contribute toward the oncogenic potential of cyclin E. Most importantly, cyclin E protein levels have been shown to correlate with a more aggressive tumor phenotype and adverse prognosis in a number of malignancies including breast, ovarian, gastric, non-small cell, and adrenocortical carcinomas, as well as non-Hodgkin's lymphoma (6–15).

The major form of deregulation of cyclin E is at the level of protein. In examining cyclin E deregulation in breast cancer, we have previously published that irrespective of whether or

not the gene is amplified (which occurs only in 10% of all breast cancer cases), the protein is independently deregulated. In fact, we have shown that in both normal and tumor cells, at the level of RNA, cyclin E is present as multiple splice variants; however, these splice variants do not give rise to protein products (16). Therefore, it seems that the primary process that contributes to deregulation of cyclin E is through posttranslational proteolytic cleavage of the full-length protein by elastase, which results in generation of the low molecular weight (LMW) forms (17).

In ovarian cancer, cyclin E gene amplification has been described in 12% to 21% of ovarian tumors (7, 18) with RNA overexpression reported in as many as 30% of cases (7, 18, 19) and up to 70% of tumors are reported to show overexpression at the protein level (20, 21). Of note, all the studies of cyclin E protein expression have relied on immunohistochemical techniques that may not be as sensitive as Western blot analysis for the overexpression and detection of the LMW form of this cell cycle regulator (12). Cyclin E expression and its link to patient outcomes in ovarian carcinoma has been investigated in a handful of studies (8, 14, 21–23). The majority of these reports show that cyclin E is an important mediator of survival in ovarian cancer (8, 14, 21, 23). None of the studies on cyclin E in ovarian cancer have evaluated the effect of cyclin E – associated kinase activity on clinical end points. This is particularly significant because protein expression may not necessarily translate into function, especially in proteins whose function is to catalytically activate a process, in this case the G₁-S transition.

Authors' Affiliations: Departments of ¹Surgical Oncology, ²Gynecologic Oncology, ³Bioinformatics and Computational Biology, and ⁴Experimental Radiation Oncology, University of Texas M. D. Anderson Cancer Center, Houston, Texas

Received 1/18/07; revised 4/19/07; accepted 5/16/07.

Grant support: National Cancer Institute grant SR01CA87548 (K. Keyomarsi) and 5P50CA116199-project 2 (G. Hortobagyi and K. Keyomarsi) and Department of Defense award DAMD17-03-1-0259.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Khandan Keyomarsi, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 066, Houston, TX 77030. Phone: 713-792-4845; Fax: 713-794-5369; E-mail: kkeyomar@mdanderson.org.

©2007 American Association for Cancer Research.

doi:10.1158/1078-0432.CCR-07-0142

Additionally, in contrast to the prognostic studies of cyclin E in cancer, there is scant information as to its role as a predictor of response to systemic therapy. In breast cancer the presence of deregulated cyclin E has been shown to be a predictor of resistance to antiestrogen therapy, primarily as a result of resistance to inhibition by p21 and p27 (3). Additionally, we have previously reported that overexpression of the deregulated LMW isoforms of cyclin E in an *in vitro* ovarian cancer cell line model provides these cells with a proliferative advantage and resistance to inhibition by p21 and p27 (2). Cyclin E overexpression in this ovarian cancer model system also increased sensitivity to cisplatin treatment. We therefore hypothesized that cyclin E overexpression, by abrogating the G₁ checkpoint and increasing the proliferative fraction, would make tumor cells more susceptible to S-phase targeted therapies, such as cisplatin. In addition, because the biological functions of cyclin E is effected in part through its associated kinase activity, we further hypothesized that cyclin E overexpression and, thus, enhanced associated kinase activity would also predict for response to platinum-based therapy. We tested this hypothesis clinically in a cohort of 75 patients with advanced ovarian carcinoma who underwent optimal surgical debulking followed by platinum-based chemotherapy. Because the major form of deregulation of cyclin E is at the level of protein, we measured cyclin E protein levels as the variable of interest to compare with our clinical end points.

Materials and Methods

Study population. Eighty-seven patients were identified from the institutional tumor bank who met the following criteria: (a) high-grade ovarian epithelial malignancy, (b) stage III/stage IV disease, (c) optimally debulked, (d) completed adjuvant platinum-based systemic therapy, and (e) did not receive neoadjuvant chemotherapy. Protein extracts were successfully generated from 81 of these patients. A further six patients were excluded due to lack of complete clinical information for a final assessable cohort of 75 patients. Patients were diagnosed between March 1993 and November 2003. In the majority of patients (72 patients), tissue was collected during surgical debulking done at presentation. Three patients had tissue collection at time of surgery for recurrent disease and had already received their platinum-based chemotherapy at time of tissue acquisition for this study. We opted to include these patients in our analysis based on our experience with breast cancer tissue cyclin E analysis before and after chemotherapy, in which we have found high concordance between pretreatment and posttreatment cyclin E levels.⁵ Clinical data and patient outcome information were collected from chart review and tumor registry information. Institutional Review Board approval was obtained for this retrospective analysis.

Chemotherapy treatment. All patients in this study received platinum-based primary therapy with either cisplatin (12 patients, 16%) or carboplatin (63 patients, 84%). In the majority of cases this was given in combination with taxanes (62 patients, 83%). Additional combinations with platinum salts included topotecan/carboplatin (3 patients), cyclophosphamide/carboplatin (2 patients), carboplatin/Taxol/herceptin (2 patients), and carboplatin/Taxol/doxil (1 patient). Five patients received carboplatin alone. The average and median number of cycles of platinum agent were both 6 (range, 1-9). Fifteen patients received further consolidation therapy after completing primary therapy. Because consolidation therapy was not given as standard treatment to all

patients, completion of primary therapy was used as the end point for determining response outcomes.

Tissue collection and processing. All tumor samples were collected according to a standardized process for banking samples in the ovarian tumor bank. This process assures that all operative samples are processed by pathology, and samples to be banked are placed in liquid nitrogen promptly after arrival in the pathology suite. For purposes of this study, all tumor samples were obtained from this institutional tumor bank depository. We have previously examined the stability of cyclin E-associated kinase activity from fresh and snap frozen clinical tumor samples by comparing the cyclin E-associated kinase activity in the tumor sample initially 2 to 3 h after harvest and again after an overnight freeze-thaw and found no differences in the functionality of cyclin E after the freezing process.⁵

Tumor samples were dissected and manually minced into small segments in a cocktail of protease inhibitors to prevent protein degradation, then further homogenized by sonication. After high-speed centrifugation, the clear lysate supernatant was removed and assayed for total protein content.

Western blot analysis. Samples were subjected to Western blot analysis as previously described (24). Briefly, 50 µg of protein from each condition was electrophoresed in each lane of a 10% (cyclin E, actin) or 13% (p21, p27, and actin) SDS-PAGE and transferred to Immobilon P for 2 h at 4°C at 85 mV constant volts. The blots were blocked overnight at 4°C in Blotto [5% nonfat dry milk in 20 mmol/L Tris, 137 mmol/L NaCl, 0.25% Tween (pH 7.6)]. After six 10-min washes in TBST [20 mmol/L Tris, 137 mmol/L NaCl, 0.05% Tween (pH 7.6)], the blots were incubated in primary antibodies for 2 h. Primary antibodies used were cyclin E HE-12 at 0.2 µg/mL (Santa Cruz Biochemicals), monoclonal antibody to p27 (Transduction Laboratories), monoclonal antibody to p21 (Oncogene Research Products/Calbiochem), and actin monoclonal antibody (Roche Molecular Biochemicals), all at 1 µg/mL in Blotto. After primary antibody incubation, the blots were washed and incubated with goat anti-mouse horseradish peroxidase conjugate at a dilution of 1:5,000 in Blotto for 1 h and finally washed and developed with the Renaissance chemiluminescence system as directed by the manufacturers (NEN Life Sciences Products).

Densitometric analysis. Western blot analysis was quantified by densitometry using IDScan EX for Windows v3.1 (Scanalytics, Inc.). For each gel, cell line extracts were included to serve as positive controls specific to the protein of interest (i.e., OVCAR-3 for cyclin E, MCF-7 for p21 and p27). These positive controls were used to allow accurate cross-comparison of cyclin E, p21, and p27 across all the different gels. Multiple different image exposures were taken for each gel. Image exposures for gels to be cross-compared were selected, such that densitometric values for the positive controls on each gel were within 20%. Ponceau staining was used to correct for protein loading. Cyclin E levels were determined as the combined densitometric value of the full-length and LMW protein levels, thereby arriving at a single value representative of the total cyclin E expression level for the sample. We chose to use total cyclin E expression rather than independently using full-length and LMW levels of the protein because our previous data had showed that the total cyclin E content was most biologically relevant when measuring clinical outcomes (12).

Cyclin E-associated kinase assay. For the cyclin E-associated kinase assay, 300 µg of protein per sample was incubated with polyclonal antibody to cyclin E as previously described (24). The immunoprecipitates were pulled down using Sepharose A beads (Amersham Biosciences), washed, and subsequently incubated with kinase assay buffer containing 60 µmol/L cold ATP and 5 µCi of [³²P]γ-ATP and 5 µg histone H1 as substrate (Roche Molecular Biochemicals) in a final volume of 30 µL at 37°C for 30 min. The products of the reaction were then analyzed on a 13% SDS-PAGE gel. The gels were then stained, destained, dried, and imaged using the Typhoon phosphor-imager system (GE Healthcare Life Sciences). The bands corresponding

⁵I. Bedrosian, unpublished observations.

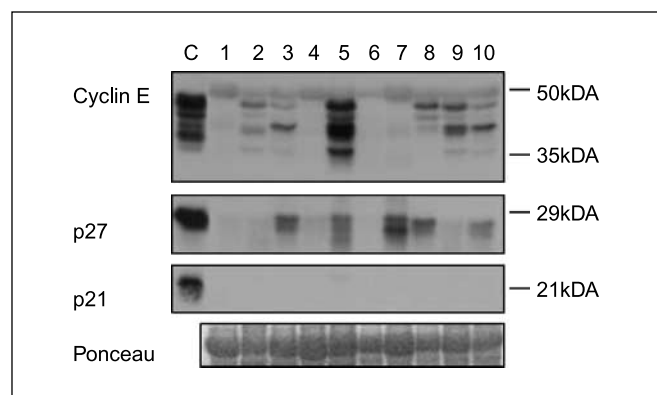


Fig. 1. Representative analysis of cyclin E expression in ovarian tumor tissue. Tumor sample lysates (50 µg) were resolved on an SDS-PAGE gel, and the membranes probed with the antibodies indicated. An association with p27 is evident, although p21 levels seemed to be uniformly low/lost across all samples investigated. C represents control samples, OVCAR for cyclin E blots and MCF-7 for p21 and p27 blots. Ponceau staining of the blot was used as a measure of equal loading of protein on each lane of the SDS-PAGE.

to histone H1 were quantified. A tumor sample with high cyclin E-associated activity was used as a positive control. This sample was included on each gel and was used as an internal reference to standardize cyclin E-associated kinase activity of the samples across all gels. The relative cyclin E-associated kinase activity of each tumor sample was calculated as a percentage of the positive control sample to obtain a relative kinase score.

To determine the relevance of cyclin E-associated kinase activity to the clinical end points of interest, relative kinase activity per sample was further normalized to the level of cyclin E within each sample as determined by densitometry, giving a kinase/cyclin E score. Unlike most established cell lines, either normal or tumor, which proliferate in culture, normal tissue has low level of proliferation. As a result of low level of proliferation, all kinase activities would also be very low and not suitable for use as a control. Our intent in this study was to establish a cyclin E kinase value relative to the amount of cyclin E in the sample to determine a kinase per unit of cyclin E for each tumor sample. We felt that this would be the most representative of the true cyclin E kinase activity for each sample and also provide a means of comparison across the samples in this study.

Statistical analysis. Spearman analysis was used to test for correlations between cyclin E expression and patient age at diagnosis, expression level of p27, and relative cyclin E-associated kinase activity. The Mann-Whitney two-sample test was used to compare the distributions of cyclin E expression levels in subgroups of patients by stage (III versus IV) or histology (serous versus other). Univariate and multivariate logistic regression was used to investigate the association with clinical response to platinum-based chemotherapy of each of the variables described above. Response to therapy was defined as per the 2003 revision of the Gynecologic Oncology Group criteria. Patients who progressed on chemotherapy or who relapsed within 6 months of completing primary therapy were deemed to be nonresponders. All other patients were categorized as responders to chemotherapy.

Time-to-tumor recurrence was calculated from the end of primary treatment to the date of documented progression or recurrence. Patients not experiencing recurrence were censored at last follow-up for the recurrence end point. Survival was calculated from time of completion of primary therapy to date of last follow-up. Patients alive at last follow-up were censored for the survival end point. The Cox proportional hazards model was used to test for differences in freedom from recurrence (FFR) and survival according to clinical factors or molecular characteristics of disease.

Given the wide range of values for cyclin E expression levels, relative cyclin E-associated kinase activity, kinase/cyclin E ratios and p27 expression levels, each of these variables was converted to a log₁₀ scale for all analyses.

Results

Association between cyclin E and clinical, histologic, and molecular variables. A representative panel of tumor samples and their expression of cyclin E, p27, and p21 by Western blot analysis are shown in Fig. 1. Overexpression of cyclin E seen in the majority of tumors (84%) correlated to the appearance of the LMW forms of the protein and also seemed to correspond to higher expression of p27 (Fig. 2). p21 levels were undetectable in nearly all ovarian tumors in this study, and therefore, no further analysis with this variable was done.

Cyclin E expression levels as measured by densitometry ranged from 2.2×10^4 to 2.7×10^6 units with a median value of 5.0×10^5 . Cyclin E levels were independent of age, tumor stage, and histology (Table 1). Increasing cyclin E levels did however correlate significantly with increasing p27 levels ($P = 0.04$) although with a relatively low correlation coefficient for this association of 0.24 (Fig. 2). These data are in contrast with previous reports showing an inverse correlation between cyclin E over expression and p27 levels (21, 25–27). Therefore, the low correlation coefficient observed in our study suggests that the prognostic relevance of cyclin E overexpression may be independent of p27.

Determination of cyclin E-associated kinase activity. Sufficient sample was available from 74 of 75 patients for cyclin E-associated kinase evaluation. Cyclin E immunoprecipitates from each tumor sample were assessed for cyclin E associated kinase activity using Histone H1 as substrate. Relative kinase activity for each sample was calculated as a percentage of reference control sample. To ensure reproducibility of the assay, 11 samples were repeated at least twice. These samples were repeated on different days at least 1 week apart. The results of 10 of these samples are shown in Fig. 3. As can be seen, cyclin E-associated kinase activity, represented by phosphorylation of the histone H1 substrate, is similar across replicate samples

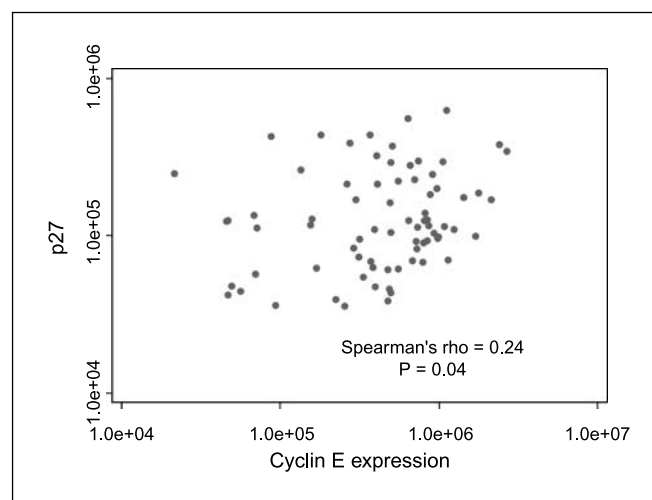


Fig. 2. A significant association is seen between increasing cyclin E and increasing p27 levels in the 75 ovarian tumor samples studied.

Table 1. Association between cyclin E expression and clinical, histologic and molecular variables

Variable	Univariate <i>P</i> value
Age (median 58 y, range 37-83 y)	0.34
Stage III (<i>n</i> = 68) versus Stage IV (<i>n</i> = 7) disease	0.09
Histology: serous (<i>n</i> = 60) versus other (<i>n</i> = 15)	0.32
p27 (median 1.2×10^5 , range 3.5×10^4 to 6.3×10^5)	0.04
Relative cyclin E-associated kinase activity (median 53.2%, range 8.9-382%)	0.58

despite being done on different days. Sample means and SD were also calculated for each replicate sample, and the coefficient of variation was computed. The average coefficient of variation was 9.9% indicating high reproducibility. These data supports the robustness of the cyclin E-associated kinase assay and indicate that the results obtained are reliable measures of the cyclin E-associated kinase activity of tumor samples.

Association between cyclin E expression and its associated kinase activity. We next examined the correlation between cyclin E expression level and its associated kinase activity in the 74 patients for whom both data points had been obtained. As described above, relative cyclin E kinase activity for each sample was calculated as a function of the control specimen tested on each gel. We found no statistically significant correlation between cyclin E protein level in the tumor samples and relative cyclin E-associated kinase activity ($P = 0.58$; Table 1; Fig. 4). This result shows the complexity of kinase activity, which depends not only on the protein level of cyclin E, but also on the levels of its kinase binding partner, cdk2, expression of the natural inhibitors, p21 and p27 (Fig. 5) as well as other kinase binding partners, some known (such as cdk1) and others unknown to date. Thus, examining just the level of cyclin E protein may provide an incomplete assessment of the function of this protein.

Response to chemotherapy. Clinical data on all 75 patients was available to determine response to platinum-based chemotherapy. Using the GOG criteria (see Materials and Methods), 21 patients were identified as nonresponders and 54 were responders, including 16 patients who never recurred during the follow-up period; median follow-up for all patients was 28 months (range, 1-125 months). All patients classified as responders had a minimum of 6 months of follow-up after completion of primary therapy. We investigated the association of clinical variables, as well as expression levels of cyclin E and p27, and cyclin E-associated kinase activity to clinical response outcomes. In univariate analyses, the only predictor of response was the cyclin E-associated kinase activity of the tumor sample [normalized to cyclin E (kinase/cyclin E ratio) as described in Materials and Methods], with low normalized kinase activity predicting for a lesser likelihood of response (Table 2; Fig. 6). Neither cyclin E protein levels nor p27 expression levels were found to be predictive of response. All the variables assessed by univariate analysis were next tested in a multivariate logistic regression model. Again, the only factor that significantly

predicted response to platinum-based chemotherapy was the cyclin E-associated kinase activity. This result underscores the importance of evaluating cyclin E-associated kinase activity as a variable independent of protein level expression.

FFR and overall survival. We next used the Cox proportional hazards ratio to investigate the association between clinical and molecular factors and FFR. For continuous variables in this analysis (age, cyclin E levels, p27 levels, and ratio of cyclin E-associated kinase/cyclin E protein level), the median value was used to divide patients into subgroups for comparison. As seen in Table 3, only cyclin E protein levels were significant predictors for FFR with a hazard ratio of 2.0 (95% confidence interval, 1.2-3.4; $P = 0.01$). Stage IV disease approached significance with a P value of 0.06. Interestingly, cyclin E-associated kinase activity did not predict for FFR ($P = 0.25$). Age, histology (serous versus nonserous), and p27 expression level also had no effect on FFR.

Overall survival data is shown in Table 4. The only variable that predicted for overall survival was Stage IV disease (hazard ratio, 5.9; 95% confidence interval, 2.3-15.2; $P < 0.01$). Neither cyclin E nor cyclin E-associated kinase activity was predictive of overall survival. This is not unexpected, given the fewer events in this analysis which limits statistical power. In addition, the heterogeneity of salvage therapies instituted after relapse also likely makes it difficult to directly test the relevance of cyclin E on disease biology and survival.

Discussion

The role of cyclin E in oncogenesis and its function as a clinical prognostic indicator in cancer patients have been the focus of many reports in recent years. Our increasing understanding of the biology of deregulated cyclin E has highlighted the diverse functions of this protein, independent of its role in regulating the G₁-S checkpoint and independent of its primary kinase partner, cdk2. By investigating the roles of cyclin E protein and its associated kinase activity in predicting clinical outcomes, such as response to therapy, our data provide the first clinical evidence of the diversity of cyclin E's role in the oncogenic process and provide important translational support for cyclin E functions independent of its associated kinase activity.

Based on our preclinical models (2), we had anticipated that cyclin E levels would be predictive of higher levels of cyclin

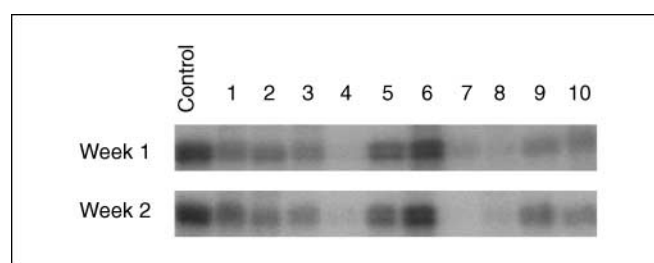


Fig. 3. Cyclin E-associated kinase assay shows high reproducibility. Cyclin E-associated kinase activity was repeated at least twice at least 1 wk apart. Relative kinase activity for each sample was calculated as a percentage of reference control sample. The control sample represents an ovarian tumor specimen with high levels of cyclin E activity. Cyclin E-associated kinase activity for each sample was calculated as a function of control. Mean cyclin E-associated kinase activity and SD for each replicate sample was calculated. The average coefficient of variation was 9.9% indicating high reproducibility.

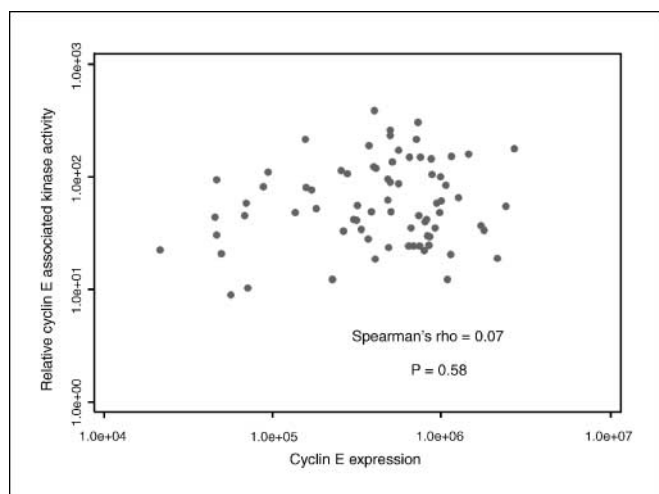


Fig. 4. No correlation is seen between cyclin E protein levels and its associated kinase activity.

E-associated kinase activity and would also identify patients with higher propensity to respond to S-phase targeted therapy, such as cisplatin and carboplatin. We were therefore surprised to find that cyclin E protein levels did not correlate with cyclin E-associated kinase activity and had no predictive value on response variables. Rather, the direct measure of cyclin E-associated kinase activity, which is likely a measure of higher proliferative activity, was the only predictive marker of platinum sensitivity. This decoupling of cyclin E protein level from that of its associated kinase activity implies that cyclin E has additional functions, independent of its associated kinase activity.

There are few reports of cyclin E activities that are specifically separate from its partnership with cyclin-dependent kinases. A suggestion that cyclin E may have important biological roles that are independent of its ability to bind and activate cdk2 comes from studies in which cdk2 has been shown to be dispensable for tumor formation in p27 null animals, but cyclin E-deficient cells are relatively resistant to oncogenic transformation (28). More direct evidence comes from recent

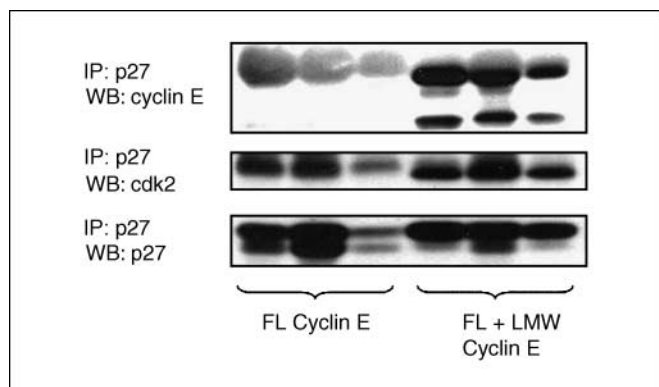


Fig. 5. Cyclin E complexes with cdk2 and p27. Tumor lysate (300 µg) was immunoprecipitated (IP) using a polyclonal antibody to p27, and the complex pulled down using sepharose beads. The complex was then denatured and resolved on a 10% (cyclin E) or 13% (cdk2, p27) SDS-PAGE gel. The membranes were probed (WB) with the antibodies indicated. The results confirm the recognized interaction between cyclin E, cdk2, and p27.

Table 2. Univariate analysis of predictors of response to primary therapy

Variable	P*
Age	0.99
Stage (III versus IV)	0.97
Histology (serous versus other)	0.90
Cyclin E expression	0.20
p27 expression	0.53
Kinase activity/cyclin E	0.045

*Logistic regression.

data that cyclin E has a centrosomal localization signal that is independent of its kinase function (29). Whether additional roles of deregulated cyclin E, such as induction of chromosomal instability (4) and activation of DNA damage checkpoints (30), are independent of cyclin E-associated kinase activity are unknown and subject to further testing.

Our finding in this study that cyclin E and p27 levels increase concordantly is in contrast with other reports (21, 25–27). It has been suggested in these studies that the high cyclin E levels in conjunction with low p27 levels is the primary predictor of poor clinical outcome in patients with cyclin E-overexpressing tumors. The discrepancy between our data and other reports is difficult to reconcile. The most likely reasons are differences in assay systems. First, our use of Western blot assays likely increases the sensitivity of detection of these proteins. Second, we did not determine cut points or assign high/low groupings when comparing the association between these two markers, instead choosing to compare them as continuous variables, which is a more accurate representation of true differences than arbitrary cut-off points.

Consistent with other reports, our study shows that cyclin E protein levels were predictive of patient outcomes measured in our series as a function of FFR. We chose to use primarily the FFR end point for the following reasons: (a) more events were

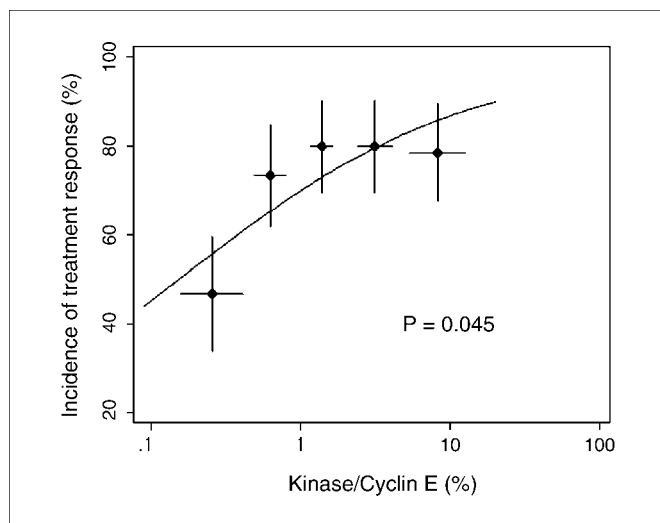


Fig. 6. Cyclin E-associated kinase levels correlate with probability of response to platinum-based chemotherapy.

Table 3. Cox proportional hazards FFR analysis

Variable	HR (95% CI)	P
Age (>58 y; n = 35)	1.3 (0.8-2.2)	0.34
Stage IV (n = 7)	2.2 (1.0-4.9)	0.06
Serous histology (n = 47)	1.0 (0.5-1.8)	0.95
Cyclin E expression (>496,553; n = 37)	2.0 (1.2-3.4)	0.01
p27 (>115,448; n = 37)	1.1 (0.6-1.8)	0.82
Kinase/cyclin E ratio (<1.32 × 10 ⁻⁴ ; n = 37)	1.4 (0.8-2.3)	0.25

Abbreviations: 95% CI, 95% confidence interval; hazard ratio, HR.

Table 4. Cox proportional hazards overall survival analysis

Variable	HR (95% CI)	P
Age (>58; n = 35)	0.7 (0.3-1.5)	0.39
Stage IV (n = 7)	5.9 (2.3-15.2)	<0.01
Serous histology (n = 47)	1.1 (0.4-2.8)	0.88
Cyclin E expression (>496,553; n = 37)	1.5 (0.7-3.1)	0.31
p27 (>115,448; n = 37)	0.9 (0.4-1.8)	0.75
Kinase/cyclin E ratio (<1.32 × 10 ⁻⁴ ; n = 37)	0.5 (0.2-1.1)	0.09

available for FFR, therefore providing more statistical power for the analysis (60 patients had experienced recurrences in the follow-up period, with only 32 of these patients having died) and (b) the FFR end point does not reflect the effects of any salvage therapy instituted after relapse and, hence, provides a more direct evaluation of cyclin E overexpression on tumor biology and behavior. However, we also report on the novel finding that cyclin E-associated kinase activity had no prognostic relevance. Our data therefore imply a compartmentalization of cyclin E's oncogenic functions, with kinase activity likely reflecting the proliferative advantage conferred to the tumor cell and determining response to chemotherapy and kinase-independent activity promoting additional biological processes that effect survival outcomes. These kinase-independent activities of cyclin E remain unknown at present but may include cyclin E's role in mediating oncogenic stress and genomic instability. Our observation on the separate roles of cyclin E and cyclin E-associated kinase activity also explains why cyclin E protein expression has not consistently correlated with measures of proliferation, such as ki-67. We would anticipate that such a correlation would be better assessed by investigating the association between proliferation and cyclin E-associated kinase activity.

As agents targeting cyclin-dependent kinases undergo clinical trials, the results from our study highlight that cyclin levels may not be an appropriate surrogate for cyclin-associated kinase activity. For instance, cyclin E activity is dependent on multiple factors in addition to the level of cyclin E expression, including expression of cdk2, p21, and p27. It is the sum of these components that determines the ultimate activity of the cyclin E-cdk complex, which mediates

the transition across the restriction point into S phase. Our data therefore suggests that therapies that target the cyclin-dependent kinase activity may need to select patients on the basis of kinase function rather than expression of the target protein. An additional implication of our findings is that even successful targeting of cyclin-dependent kinase pathways may not alter the ultimate clinical outcome (disease recurrence) of patients with ovarian cancer.

Our study used tissue collected from patients as part of a prospective protocol for tissue banking at our institution. Although patients enrolled are prospectively identified, enrollment and tissue acquisition is predicated on a number of factors. First, patients have to acquiesce to enrollment and tumor collection. The second is the determination by the pathologist that a portion of the tumor can be banked without compromising pathologic evaluation. Therefore, even in a prospective collection process, patient and pathologist influences may bias the collection process and potentially the results obtained. It is difficult to speculate how such biases may effect the results reported. Therefore, validation of our findings using samples from other banked sources will be important.

In summary, our study makes the novel observation that cyclin E protein and cyclin E-associated kinase activity have distinct roles on clinical outcome measures in ovarian cancer patients. These data add to our understanding of the complexity of cyclin E deregulation on the oncogenic process and suggests that cyclin E has functions independent of its associated kinase activity that are determinants of survival. A better understanding of these kinase-independent activities mediated by cyclin E deregulation is critical to be able to meaningfully target cyclin E and thus effect the long-term prognosis of patients with cyclin E overexpressing tumors.

References

- Ohtsubo M, Roberts J. Cyclin-dependent regulation of G₁ in mammalian fibroblasts. *Science* 1993;259:1908-12.
- Bedrosian I, Lu KH, Verschraegen C, Keyomarsi K. Cyclin E deregulation alters the biologic properties of ovarian cancer cells. *Oncogene* 2004;23:2648-57.
- Akli S, Zheng PJ, Multani AS, et al. Tumor-specific low molecular weight forms of cyclin E induce genomic instability and resistance to p21, p27, and antiestrogens in breast cancer. *Cancer Res* 2004;64:3198-208.
- Spruck CH, Won KA, Reed SI. Deregulated cyclin E induces chromosome instability. *Nature* 1999;401:297-300.
- Lengauer C, Kinzler KW, Vogelstein B. Genetic instability in colorectal cancers. *Nature* 1997;386:623-7.
- Bani-Hani KE, Almasri NM, Khader YS, Sheyab FM, Karam HN. Combined evaluation of expressions of cyclin E and p53 proteins as prognostic factors for patients with gastric cancer. *Clin Cancer Res* 2005;11:1447-53.
- Courjal F, Louason G, Speiser P, Katsaros D, Zeilinger R, Theillet C. Cyclin gene amplification and overexpression in breast and ovarian cancers: Evidence for the selection of cyclin D1 in breast and cyclin E in ovarian tumors. *Int J Cancer* 1996;69:247-53.
- Farley J, Smith LM, Darcy KM, et al. Cyclin E expression is a significant predictor of survival in advanced, suboptimally debulked ovarian epithelial cancers: a Gynecologic Oncology Group study. *Cancer Res* 2003;63:1235-41.
- Ferreri AJ, Ponzoni M, Pruneri G, et al. Immunoreactivity for p27(KIP1) and cyclin E is an independent predictor of survival in primary gastric non-Hodgkin's lymphoma. *Int J Cancer* 2001;94:599-604.
- Fukue T, Hirata T, Naiki H, Hitomi S, Wada H. Prognostic significance of cyclin E overexpression in resected non-small cell lung cancer. *Cancer Res* 2000;60:242-4.
- Jang SJ, Park YW, Park MH, et al. Expression of cell-cycle regulators, cyclin E and p21WAF1/CIP1, potential

- prognostic markers for gastric cancer. *Eur J Surg Oncol* 1999;25:157–63.
12. Keyomarsi K, Tucker SL, Buchholz TA, et al. Cyclin E and survival in patients with breast cancer. *N Engl J Med* 2002;347:1566–75.
13. Porter PL, Malone KE, Heagerty PJ, et al. Expression of cell-cycle regulators p27Kip1 and cyclin E, alone and in combination, correlate with survival in young breast cancer patients. *Nat Med* 1997;3:222–5.
14. Rosen DG, Yang G, Deavers MT, et al. Cyclin E expression is correlated with tumor progression and predicts a poor prognosis in patients with ovarian carcinoma. *Cancer* 2006;106:1925–32.
15. Tissier F, Louvel A, Grabar S, et al. Cyclin E correlates with malignancy and adverse prognosis in adrenocortical tumors. *Eur J Endocrinol* 2004;150:809–17.
16. Porter D, Keyomarsi K. Novel splice variants of cyclin E with altered substrate specificity. *Nucl Acids Res* 2000;28:E101.
17. Porter D, Zhang N, Danes C, et al. Tumor Specific Proteolytic Processing of Cyclin E Generates Hyper-Active Lower Molecular Weight Forms. *Mol Cell Biol* 2001;21:6254–69.
18. Marone M, Scambia G, Giannitelli C, et al. Analysis of Cyclin E and CDK2 in ovarian cancer: gene amplification and RNA overexpression. *Int J Cancer* 1998;75:34–9.
19. Sawasaki T, Shigemasa K, Shiroyama Y, et al. Cyclin E overexpression in epithelial ovarian cancers: inverse correlation with p53 protein accumulation. *J Soc Gynecol Invest* 2001;8:179–85.
20. Shimizu M, Nikaido T, Toki T, Shiozawa T, Fujii S. Clear Cell Carcinoma Has an Expression Pattern of Cell Cycle Regulatory Molecules that is Unique among Ovarian Adenocarcinomas. *Cancer* 1999;85:669–77.
21. Sui L, Dong Y, Ohno M, et al. Implication of Malignancy and Prognosis of p27 (kip1), Cyclin E and CDK2 Expression in Epithelial Ovarian Tumors. *Gynecol Oncol* 2001;83:56–63.
22. Milde-Langosch K, Hagen M, Bamberger AM, Loning T. Expression and prognostic value of the cell-cycle regulatory proteins, Rb, p16MTS1, p21WAF1, p27KIP1, cyclin E, and cyclin D2, in ovarian cancer. *Int J Gynecol Pathol* 2003;22:168–74.
23. Rosenberg E, Demopoulos R, Zeleniuch-Jacquette A, et al. Expression of cell cycle regulators p57 (KIP2), cyclin D1 and cyclin E in epithelial ovarian tumors and survival. *Human Pathol* 2001;32:808–13.
24. Rao S, Lowe M, Herliczek T, Keyomarsi K. Lovastatin mediated G₁ arrest in normal and tumor breast cells is through inhibition of CDK2 activity and redistribution of p21 and p27, independent of p53. *Oncogene* 1998;17:2393–402.
25. Chappuis PO, Donato E, Goffin JR, et al. Cyclin E expression in breast cancer: predicting germline BRCA1 mutations, prognosis and response to treatment. *Ann Oncol* 2005;16:735–42.
26. Goto Y, Kawauchi S, Ihara K, et al. The prognosis in spindle-cell sarcoma depends on the expression of cyclin-dependent kinase inhibitor p27 (Kip1) and cyclin E. *Cancer Sci* 2003;94:412–7.
27. Han S, Park K, Bae BN, et al. Prognostic implication of cyclin E expression and its relationship with cyclin D1 and p27Kip1 expression on tissue microarrays of node negative breast cancer. *J Surg Oncol* 2003;83:241–7.
28. Geng Y, Yu Q, Sicinska E, et al. Cyclin E ablation in the mouse. *Cell* 2003;114:431–43.
29. Matsumoto Y, Maller JL. A centrosomal localization signal in cyclin E required for Cdk2-independent S phase entry. *Science* 2004;306:885–8.
30. Bartkova J, Horejsi Z, Koed K, et al. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature* 2005;434:864–70.