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14. ABSTRACT The deregulation of cell cycle checkpoints, with loss of regulation at the G1/S transition, has been shown to play an important role in the transformation to a malignant phenotype. Our studies have focused on cyclin E, which appears in late G1 and flanks the restriction point. We hypothesize that alterations of cyclin E in ovarian cancer cells contributes to the oncogenesis of ovarian tumors and negatively impacts outcome in patients with Stage I-III cancer. In this proposal we will i) develop a comprehensive ovarian cell line model for characterization of the role of cyclin E in ovarian cancer, ii) delineate the role of cyclin E and its tumor specific LMW forms in the development of malignant phenotype in vitro and in nude mice. iii) establish the prognostic value of the hyperactive forms of cyclin E in patients with Stage I-III ovarian cancer and iv) examine the biochemical significance of the LMW forms of cyclin E in tumor specimens. The results from our studies will provide much needed information about the molecular biology of ovarian carcinoma and may open new avenues for the development of targeted therapies.					
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Introduction:

The overall purpose of this 3 year study is to test the hypothesis that alterations of cyclin E in ovarian cancer cells contributes to the oncogenesis of ovarian tumors and negatively impacts outcome in patients with Stage I-III ovarian cancer. We have accomplished all the aims of the study and our findings were recently published in the journal Clinical Cancer Research. Below is a detailed description of this study.

Over expression of the cyclin E protein has been linked to shortening of 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 towards 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 appears that primary process that contributes to deregulation of cyclin E is through post-translational 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 12-21% of ovarian tumors^(7, 18) with RNA over expression reported in as many as 30% of cases^(7, 18, 19) and up to 70% of tumors are reported to show over expression 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 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 impact of cyclin E associated kinase activity on clinical endpoints. This is particularly significant since protein expression may not necessarily translate into function, especially in proteins whose function is to catalytically activate a process, in this case the G1 to S transition.

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 anti-estrogen therapy primarily as a result of resistance to inhibition by p21 and p27.⁽³⁾ Additionally, we have previously reported that over expression 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 27.⁽²⁾ Cyclin E overexpression in this ovarian cancer model system also increased sensitivity to cisplatin treatment. We therefore hypothesized that cyclin E over expression, 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, since the biologic functions of cyclin E is effected in part through its associated kinase activity,

we further hypothesized that cyclin E over expression 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. Since 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 to our clinical endpoints.

In the last year of the proposed grant we have completed the last aim/task of this grant application and have also published our work. [Cyclin E-associated kinase activity predicts response to platinum-based chemotherapy. Bedrosian I, Lee C, Tucker SL, Palla SL, Lu K, Keyomarsi K. Clin Cancer Res. 2007 Aug 15;13(16):4800-6.]

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 Figure 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 appeared to correspond to higher expression of p27 (Figure 2). p21 levels were undetectable in nearly all ovarian tumors in this study and therefore no further analysis with this variable was performed.

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

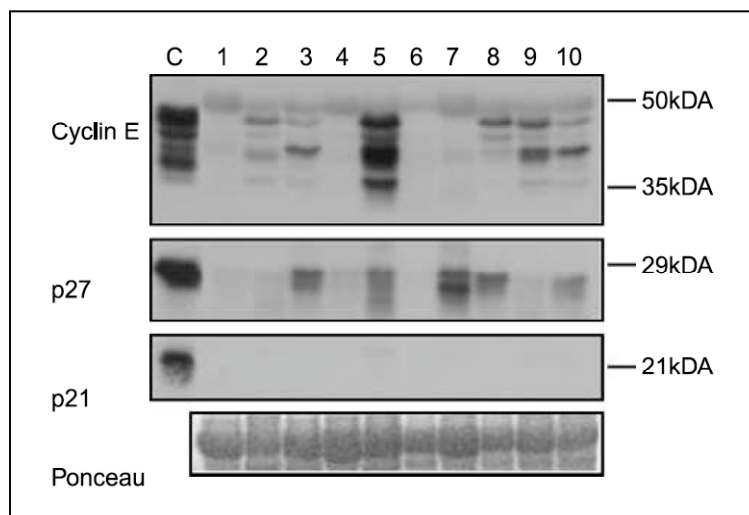


Fig. 1. Representative analysis of cyclin E expression and function in ovarian tumor tissue. Tumor sample lysates (50 μ g) were resolved on an SDS-PAGE gel, and the membranes probed with the antibodies indicated. An association with p27 is evident, although p21 levels seemed to be uniformly low/lost 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

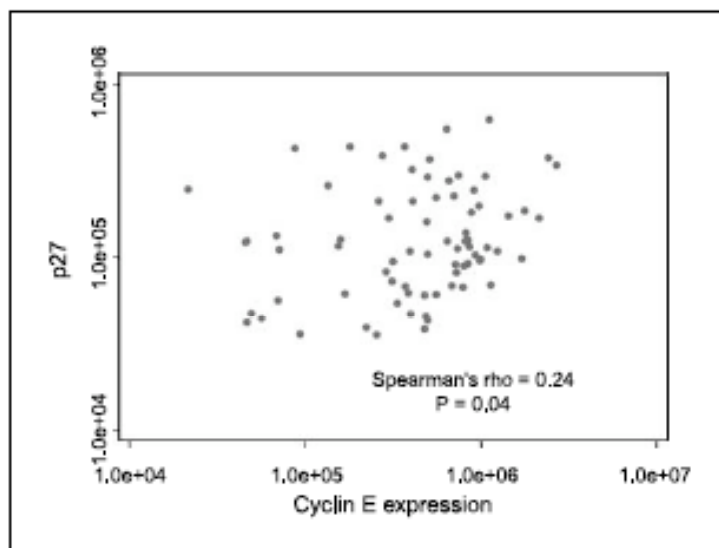


Fig. 2. A significant association is seen between increasing cyclin E and increasing p27 levels in the 75 ovarian tumor samples studied.

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

Variable	Univariate P value
Age (median 58 y, range 37-83 y)	0.34
Stage III (n = 68) versus Stage IV (n = 7) disease	0.09
Histology: serous (n = 60) versus other (n = 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

increasing p27 levels (p=0.04)

although with a relatively low

correlation co-efficient for this

association of 0.24 (Figure 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 the 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 percent of reference control sample. In order to ensure

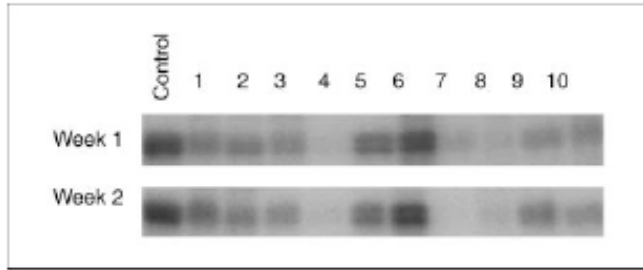
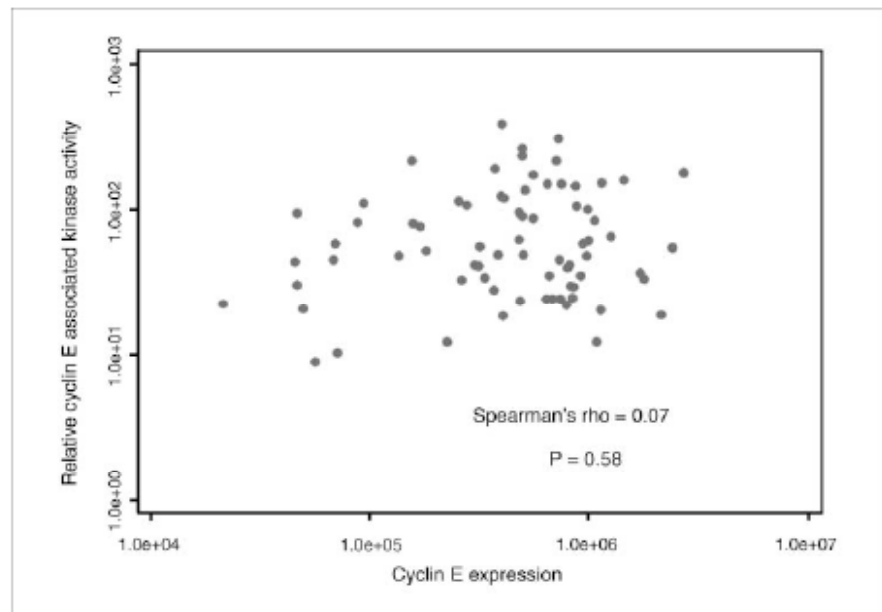


Fig. 3. Cyclin E – associated kinase assay shows high reproducibility. Cyclin E – associated kinase activity was repeated at least twice at least 1 wk apart. Relative kinase activity for each sample was calculated as a percentage of reference control sample. The control sample represents an ovarian tumor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

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 Figure 3. As can be seen, cyclin E associated kinase activity, represented by phosphorylation

of the Histone H1 substrate, is similar across replicate samples despite being performed on different days. Sample means and standard deviation were also calculated for each replicate sample and the co-efficient of variation was computed. The average co-efficient of variation was

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



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,

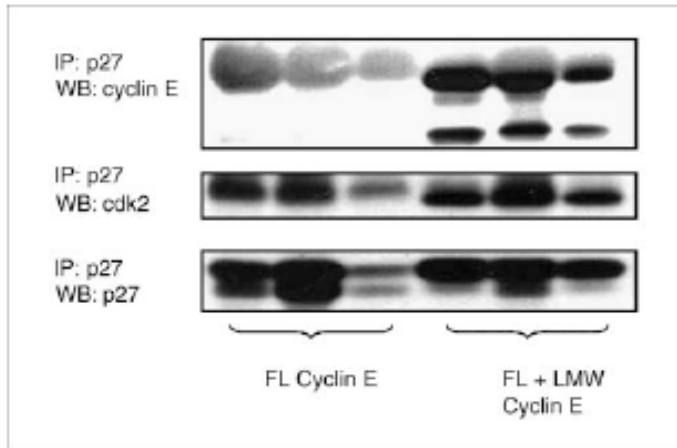


Fig. 5. Cyclin E complexes with cdk2 and p27. Tumor lysate (300 µg) was immunoprecipitated (IP) using a polyclonal antibody to p27, and the complex pulled down using sepharose beads. The complex was then denatured and resolved on a 10% (cyclin E) or 13% (cdk2, p27) SDS-PAGE gel. The membranes were probed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

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, Figure 4). This result demonstrates 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 (Figure 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 methods), 21 patients were identified as non-responders 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

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.

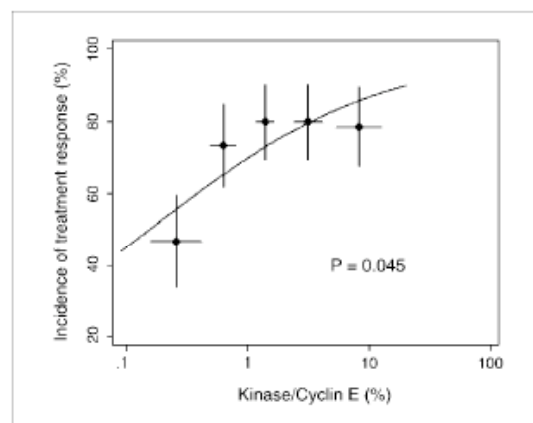


Fig. 6. Cyclin E-associated kinase levels correlate with probability of response to platinum-based chemotherapy.

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 methods), with low normalized kinase activity predicting for a lesser likelihood of response (Table 2) (Figure 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.

Freedom from Recurrence and Overall Survival

We next used the Cox proportional hazards ratio to investigate the association between clinical and molecular factors and freedom from recurrence (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 utilized 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%CI[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 vs non-serous), and p27 expression level also had no impact on FFR.

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 ($<1.32 \times 10^{-4}$; $n = 37$)	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 ($<1.32 \times 10^{-4}$; $n = 37$)	0.5 (0.2-1.1)	0.09

Overall survival data is shown in Table 4. The only variable that predicted for overall survival was Stage IV disease (HR 5.9, 95% CI [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 has 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 provides the first clinical evidence of the

diversity of cyclin E's role in the oncogenic process and provides 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 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 parameters. 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 where 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 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 rise concordantly is in contrast with other reports.^(21, 25-27) It has been suggested in these studies that it is high cyclin E levels in conjunction with low p27 levels that 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 2 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 utilize primarily the FFR endpoint for the following reasons: i) more events were available for FFR therefore providing more statistical power for the analysis. Sixty patients had experienced recurrences in the follow-up period, with only 32 of these patients having died, and ii) the FFR endpoint does not reflect the effects of any salvage therapy instituted after relapse and hence provides a more direct evaluation of cyclin E over expression 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 biologic processes that impact 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 utilized 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 impact 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. This data adds 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 impact the long term prognosis of patients with cyclin E overexpressing tumors.

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Conclusions

As presented in detail in this report-we have addressed all the tasks in this grant and our manuscript was published in *Clinical Cancer Research*, referenced here.

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