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Award Number: DAMD17-99-1-9184

TITLE: Cyclin D and Cyclin E as Markers of Therapeutic Responsiveness in Breast Cancer

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REPORT DATE: May 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2001	Annual Summary (1 May 00 - 30 Apr 01)

4. TITLE AND SUBTITLE

Cyclin D and Cyclin E as Markers of Therapeutic Responsiveness in Breast Cancer

5. FUNDING NUMBERS

DAMD17-99-1-9184

6. AUTHOR(S)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

There is now accumulating evidence to suggest that aberrant expression of cyclin D1 and cyclin E occurs in human breast cancer and overexpression of these two oncogenes is associated with poor prognosis in primary breast cancers. The role of cyclin D1 and cyclin E expression as markers of therapeutic responsiveness to cancer treatment was investigated using cell lines constitutively overexpressing cyclin D1 or cyclin E. In the first instance, we tested whether overexpression of cyclin D1 or cyclin E may result in resistance to endocrine treatment in ER-positive T-47D human breast cancer cell lines using S-phase fraction and colony-forming assays. Our findings indicated that overexpression of Cyclin D1 and to a lesser extent cyclin E confer resistance to progestin treatment. In contrast, overexpression of cyclin D1 or cyclin E appeared to interfere with the early cell cycle effects of antiestrogen, but the long-term antiestrogen-induced growth inhibition remained effective in cyclin D1 or cyclin E overexpressing breast cancer. Downregulation of cyclin D1 levels by antiestrogen results in sensitivity of the cells to the antiestrogen inhibition of cell proliferation in the long-term. Cyclin D1 overexpression leads to progestin resistance without sequestration of p27, indicating that regulation of cyclin D1 is a critical element of progestin inhibition in breast cancer cells. Colony-forming assays to date suggested that cyclin D1 or cyclin E had no impact on the sensitivity of breast cancer cells to chemotherapeutic agents including doxorubicin, methotrexate, 5-fluorouracil and paclitaxel which are commonly used in clinical breast cancer treatment. In the next 12 months, the significance of cyclin D1 or cyclin E as markers of responsiveness to endocrine and radiation treatment will be investigated in the clinical settings retrospectively. The findings from this project may aid the management of breast cancer in the short term and may contribute to identifying potential targets for modulation of drug resistance in breast cancer therapy in the long term.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, Cyclin D1, Cyclin E, Therapeutic Responsiveness	10
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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ANNUAL SUMMARY - Year 2

AWARD NUMBER: DAMD17-99-1-9184

Introduction

Cyclins belonging to the D and E families and their respective kinase partners play a pivotal role in regulating the progression of diverse cell types through G_1 phase of the cell cycle. Deregulated expression of either cyclin D1 or cyclin E can provide a growth advantage to tumor cells; their expression in the mammary gland of transgenic mice results in abnormal epithelial proliferation and adenocarcinoma, and they thus function as oncogenes. There is now accumulating evidence to suggest that aberrant expression of cyclin D1 and cyclin E occurs frequently in human breast cancer. Overexpression of these 2 genes is associated with poor prognosis in primary breast cancers. This could be in part due to reduced responsiveness of the tumors to systemic treatment. The aim of this project is to define the role of cyclin D1 and cyclin E as markers of therapeutic responsiveness in both preclinical and clinical models of breast cancer.

The project is designed to:
1. assess the relationship between cyclin D1/cyclin E expression and response to therapy including endocrine treatment, chemotherapy and radiation therapy in *in-vitro* studies.
2. investigate the relationship between the levels of cyclin D1 and cyclin E expression, response rate and survival in the clinical setting.
3. determine the underlying mechanisms contributing to cyclin D1 and cyclin E altering response to breast cancer therapies *in-vitro*

Body of Report

Task 1: *In-vitro* study to determine the relationship between cyclin D1/cyclin E expression and response to cancer therapy

Breast cancer cell lines constitutively overexpressing cyclin D1 or cyclin E were produced and characterized in the first 12 months. In months 12 - 24, these cell lines were used to address whether cyclin D1 and cyclin E overexpression alters response to breast cancer therapeutic agents *in-vitro*.

Previous *in-vitro* studies from our laboratory clearly demonstrated that ectopic induction of cyclin D1 expression in ER-positive breast cancer cell lines (T-47D and MCF-7) can overcome the inhibition of cell cycle progression induced by antiestrogen (1) suggesting indirectly that cyclin D1 overexpression may confer resistance to endocrine treatment. However, another study showed that tet-inducible cyclin D1 overexpression in MCF-7 breast cancer cells does not prevent inhibition of cell growth by antiestrogens (2). A recent clinical study from this laboratory suggested that the duration of the response to tamoxifen was significantly longer in ER-positive patients with low cyclin D1 mRNA

levels than in those with high cyclin D1 (3), implying that overexpression of cyclin D1 may confer a degree of resistance to antiestrogen therapy, although the sample size in the subgroup treated with antiestrogen in this study was small and the analyses must therefore be interpreted with caution. We therefore in the first instance tested whether overexpression of cyclin D1 or cyclin E may result in resistance to endocrine treatment in ER-positive T-47D human breast cancer cell lines.

The effects of the pure steroidal antioestrogen ICI 182780 and the progestin ORG 2058 on cell proliferation in cell lines constitutively overexpressing cyclin D1 (by 8-10 fold) or cyclin E (by 3-10 fold) were investigated using flow cytometry for S-phase fraction and clonogenic assay for long term growth effects. Treatment of cyclin D1 and cyclin E overexpressing cell lines with ICI 182780 led to partial resistance to antiestrogenic effects on cell cycle progression during the first 48 – 72 hours of treatment. Treatment of cells overexpressing cyclin D1 with ORG 2058 only resulted in a slight reduction in S-phase at 48 hours indicating marked resistance to progestin inhibition. Overexpression of cyclin E produced a similar effect to cyclin D1 overexpression, but to a lesser extent, indicating partial resistance to progestin.

Long-term effects of progestin and antiestrogen on cell growth were investigated using a colony-forming assay. The cells were treated with ICI 182780 and ORG 2058 over a range of concentrations for 3 weeks. There was a marked reduction in sensitivity to progestin treatment in the cyclin D1 overexpressing cells, with a significant number of colonies at 3 weeks. Overexpression of cyclin E conferred partial resistance, although the cells were still clearly inhibited by progestin. In contrast, there was no significant resistance to antiestrogen treatment in cyclin D1 or cyclin E overexpressing cells in the long-term clonogenic assays.

Colony-forming assays were also used to test responsiveness to a range of chemotherapeutic agents including doxorubicin, methotrexate, 5-fluorouracil and paclitaxel, which are commonly used as therapies for clinical breast cancer. These were initially tested over a wide (10^{-5} to 10^{-11}M) concentration range to establish dose-response in this system. A narrower range of drug concentration has now been defined for more detailed experiments with each drug. Results obtained to date suggest that neither cyclin D1 nor cyclin E overexpression exert any effect on the sensitivity of the breast cancer cell lines to chemotherapeutic agents and thus this avenue of investigation has not been a high priority for further experiments. Nevertheless, if time allows in the next 12 months, the experiments will be finalized for publication. Task 1 is now essentially complete.

Task 2: *In-vivo* and clinical studies to determine the relationship between cyclin D1/cyclin E expression and response to cancer therapy

This task is the priority for the next 12 months, particularly the translational component, since the data from tasks 1 and 3 suggest that the planned *in-vivo* experiments may yield negative results. Unfortunately, the accrual of paraffin-embedded tissue blocks from patients with advanced breast cancer from ANZ breast cancer trials 7802 and 8101 treated with various chemotherapeutic and endocrine regimens has been difficult. In consequence, alternative sources of tissue have been investigated. Other collaborations locally in Sydney and overseas are being negotiated to test whether cyclin D1 and cyclin E overexpression are markers of therapeutic responsiveness to endocrine and radiation treatment in well-characterized cohorts of breast cancer patients. The availability of tumor material is promising. Once all the tissue blocks are retrieved, immunohistochemical analysis of cyclin D1 and cyclin E expression in all primary tumors will be performed. The techniques of immunohistochemistry for cyclin D1 and cyclin E have been optimised in breast tumors within our laboratory.

Task 3: Study of the underlying mechanisms in determining sensitivity to cancer therapy

The mechanistic basis for the effects of cyclin overexpression on the response to endocrine treatment has been a major focus during year 2 and these studies are nearly completed. Several molecular endpoints have been identified following acute (0 - 48 hours) treatment of MCF-7 breast cancer cells with the antiestrogen ICI 182780 in other studies from our laboratory. Inhibition of cyclin D1 gene expression and consequent decline in cyclin-Cdk4 activity is an early and critical event in antiestrogen action (4, 5). Cyclin E-Cdk2 activity is also inhibited by antiestrogen treatment and this decline is dependent on the Cdk inhibitor p21 (6). Thus, experiments were performed to define the effects of cyclin D1 and cyclin E overexpression on key molecular endpoints, including phosphorylation of pRb, cyclin D1-Cdk4 activity, cyclin E-Cdk2 activity, p21 and p27 association with these complexes.

In the parent T-47D breast cancer cells and the empty vector cells, cyclin D1 gene expression is downregulated by antioestrogen and both cyclin D1 and cyclin E levels are reduced by progestin. In the cyclin D1 overexpressing cell line, cyclin D1 expression was maintained for at least 72 hours following treatment with ICI 182780 and ORG 2058. Similarly, cyclin E expression was not decreased up to 72 hours after ORG 2058 treatment of cyclin E overexpressing cell lines. Cyclin E levels were not substantially regulated by ICI 182780 in any of the cell lines.

Following treatment of the empty vector cells with ICI 182780, the total amount of pRb decreased and the hypophosphorylated form pRb predominated. Rb phosphorylation was maintained in the cyclin D1 overexpressing cell lines at 72 hours following treatment with ICI 182780. The two cyclin E overexpressing cell lines displayed an intermediate effect on pRb phosphorylation suggesting partial resistance. The cell line expressing higher

levels of cyclin E showed a greater degree of resistance. These findings are consistent with the assessment of cell proliferation by S-phase fraction after 48 hours of ICI 182780 treatment (task 1).

A substantial increase in the amount of cyclin E-Cdk2-associated p21 and p27 was demonstrated in MCF-7 breast cancer cells following treatment with antiestrogen (6). The association with the Cdk inhibitors may be altered in the presence of overexpression of cyclin D1 or cyclin E and the redistribution of cdk inhibitors may affect the kinase activity and in turn phosphorylation of pRb. Western blots of p21 and p27 immunoprecipitates indicated that p21 and p27 proteins are associated with cyclin D1 in the cyclin D1-overexpressing cells to a much larger degree than the empty vector cells. However the degree of association between cyclin E and p21 or p27 was largely unchanged in the cyclin D1-overexpressing cells, suggesting that partial resistance in the short-term is independent of the availability of p21 or p27 to bind the cyclin E-Cdk2 complex. The association between cyclin E and p21 or p27 was increased in the cyclin E-overexpressing cells as compared to the empty vector cells or the cyclin D1-overexpressing cells following ICI 182780 treatment, suggesting that this association may play a more important physiological role in the antiestrogen inhibition of cell proliferation. Further experiments are ongoing to determine the cyclin E-Cdk2 kinase activity and degree of cyclin-Cdk4-specific phosphorylation of pRb.

Western blots of lysates from cyclin D1-overexpressing cells treated with ICI 182780 for 7 and 10 days indicated that cyclin D1 protein levels were markedly reduced by antiestrogen treatment, most likely due to increased degradation of the cyclin D1 protein. The downregulation of cyclin D1 in the constitutively overexpressing cell line likely accounts for the discrepancies between the short-term and long-term effect of antiestrogen treatment. This suggests an additional novel mechanism for antiestrogen regulation of cyclin D1 which may be important in the clinical setting.

In a collaborative study within our laboratory, the effect of cyclin D1 and cyclin E overexpression on progestin treatment have also been studied. When cyclin E expression was maintained during progestin treatment, cyclin E-Cdk2 activity still decreased by 50-60%. This was likely due to $p27^{Kip1}$ association, indicating that both cyclin E downregulation and $p27^{Kip1}$ recruitment contribute to decreased cyclin E-Cdk2 activity after progestin treatment. Cell proliferation was inhibited despite the presence of cyclin E-Cdk2 kinase activity similar to that of untreated control cells not overexpressing cyclin E. In contrast, when cyclin D1 expression was maintained during progestin treatment, cell proliferation continued in the presence of ORG 2058 despite decreased cyclin E-Cdk2 activity. Progestin treatment of cyclin D1-overexpressing cells was associated with increased $p27^{Kip1}$ association with cyclin E-Cdk2, demonstrating that the ability of cyclin D1 to confer progestin resistance does not depend on sequestration of $p27^{Kip1}$. These data indicate that regulation of cyclin D1 is a critical element of progestin inhibition in breast cancer cells.

Key Research Accomplishments

- Development and characterization of ER-positive T-47D breast cancer cell lines constitutively overexpressing cyclin D1 or cyclin E.

- Demonstration that ectopic overexpression of cyclin D1 confers progestin resistance in breast cancer cells following both short and long term exposure, while cyclin E overexpression has little effect.

- Demonstration that overexpression of cyclin D1 or cyclin E interferes with the early cell cycle effects of antiestrogen, but that long-term antiestrogen-induced growth inhibition remains effective in cyclin D1 or cyclin E-overexpressing breast cancer cells.

- Identification of a potential new mechanism for downregulation of cyclin D1 by antiestrogen that may be critical in determining sensitivity to antiestrogen.

- Progestin treatment of cyclin D1-overexpressing cells was associated with p27 association with cyclin E-Cdk2, demonstrating that the ability of cyclin D1 to confer progestin resistance does not depend on sequestration of p27.

Reportable Outcomes

Development of cell lines:

Clonal lines of T-47D cells stably transfected with empty pTRE vector and pTRE vector containing cyclin D1 and cyclin E have been established. 2 clonal lines overexpressing cyclin D1, 2 clonal lines overexpressing cyclin E and 1 vector-alone control clonal line have been characterized.

Presentations:

1. PI was invited to speak in the Basic Sciences of Oncology Series at the NSW Cancer Council on:
   - Molecular biology in breast cancer
   - Endocrine therapy

2. The Fourth Leura International Breast Cancer Conference, November 15 - 19th 2000.
   Constitutive overexpression of cyclin D1 or cyclin E prevents growth-inhibitory effects of progestin and antioestrogen
   Hui, R., Lee, C. S. L., Hunter, L. J., Finney, G., Musgrove, E. A., Sutherland, R. L.

3. The 13th Lorne Cancer Conference, February 8 - 11th 2001.
   Cell cycle control in breast cancer: mechanisms of CDK inactivation by progestins
   Musgrove, E.A., Swarbrick, A., Lee, C.S.L., Hunter, L.J.K., Hui, R. and Sutherland, R. L.

4. The 13th Lorne Cancer Conference, February 8 - 11th 2001.
   Role of cyclin E in progestin inhibition of proliferation
   Hunter, L.J.K., Lee, C.S.L., Hui, R., Sutherland, R.L. and Musgrove, E.A.

Publications:

1. The effect of constitutive overexpression of cyclin D1 and cyclin E on antiestrogen sensitivity in breast cancer
   Rina Hui, Georgina Finney, Christine S. L. Lee, Elizabeth A. Musgrove and Robert L. Sutherland.
   Manuscipt in preparation

2. Cyclin D1 overexpression leads to progestin resistance in T-47D breast cancer cells without sequestration of $p27^{Kip1}$
   Elizabeth A. Musgrove, Lisa-Jane Hunter, Christine S. L. Lee, Alexander Swarbrick, Rina Hui and Robert L. Sutherland.
   Manuscript in preparation

Summary and Conclusions

The relationships between cyclin D1 or cyclin E expression and response to endocrine therapy with antiestrogen and progestin in the ER positive T-47D human breast cancer cell lines have been examined using S-phase fraction and clongenic survival assay in the last 12 months. Our findings indicated that overexpression of cyclin D1 and to a lesser extent cyclin E confer resistance to progestin treatment. In contrast, overexpression of cyclin D1 or cyclin E appeared to interfere with the early cell cycle effects of antiestrogen, but the long-term antiestrogen-induced growth inhibition remained effective in cyclin D1 or cyclin E overexpressing breast cancer. Downregulation of cyclin D1 levels by antiestrogen results in sensitivity of the cells to the antiestrogen inhibition of cell proliferation in the long-term. Cyclin D1 overexpression leads to progestin resistance without sequestration of p27, indicating that regulation of cyclin D1 is a critical element of progestin inhibition in breast cancer cells. In the next 12 months, the significance of cyclin D1 or cyclin E as markers of responsiveness to endocrine and radiation will be investigated in the clinical settings retrospectively. The findings from this project may aid the management of breast cancer in the short term and may contribute to identifying potential targets for modulation of drug resistance in breast cancer therapy in the long term.

References

1. Wilcken, NRC, Prall, OWJ, Musgrove, EA, Sutherland, RL. Inducible overexpression of cyclin D1 in breast cancer cells reverses the growth-inhibitory effects of antiestrogens. Clin. Cancer Res., *3:* 849-854, 1997.
2. Pacilio, C, Germano, D, Addeo, R, Altucci, L, Petrizzi, VB, Cancemi, M, Cicatiello, L, Salzano, S, Lallemand, F, Michalides, R, Bresciani, F, Weisz, A. Constitutive overexpression of cyclin D1 does not prevent inhibition of hormone-responsive human breast cancer cell growth by antiestrogens. Cancer Res., *58:* 871-876, 1998.
3. Kenny, FS, Hui, R, Musgrove, EA, Gee, JM, Blamey, RW, Nicholson, RI, Sutherland, RL, Robertson, JFR. Overexpression of Cyclin D1 mRNA predicts for poor prognosis in oestrogen receptor positive breast cancer. Clin. Cancer Res., *5*: 2069-2076, 1999.
4. Musgrove, EA, Hamilton, JA, Lee, CS, Sweeney, KJ, Watts, CK, Sutherland, RL. Growth factor, steroid, and steroid antagonist regulation of cyclin gene expression associated with changes in T-47D human breast cancer cell cycle progression. Mol. Cell. Biol., *13:* 3577-3587, 1993.
5. Watts, CKW, Brady, A, Sarcevic, B, deFazio, A, Musgrove, EA, Sutherland, RL. Antiestrogen inhibition of cell cycle progression in breast cancer cells is associated with inhibition of cyclin-dependent kinase activity and decreased retinoblastoma protein phosphorylation. Mol. Endocrinol., *9:* 1804-1813, 1995.
6. Carroll, JS, Prall, OWJ, Musgrove, EA, Sutherland, RL. A pure estrogen antagonist inhibits cyclin E-Cdk2 activity in MCF-7 breast cancer cells and induces accumulation of p130-E2F4 complexes characteristic of quiescence. J. Biol. Chem., *275:* 38221-38229, 2000.