View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Nottingham ePrints

Hunt, Kelly K. and Karakas, Cansu and Ha, Min Jin and Biernacka, Anna and Yi, Min and Sahin, Aysegul and Adjapong, Opoku and Hortobogyi, Gabriel N. and Bondy, Melissa L. and Thompson, Patricia A. and Cheung, Kwok-Leung and Ellis, Ian O. and Bacus, Sarah and Symmans, W. Fraser and Do, Kim-Anh and Keyomarsi, Khandan (2016) Cytoplasmic cyclin E predicts recurrence in patients with breast cancer. Clinical Cancer Research . ISSN 1557-3265

Access from the University of Nottingham repository:
http://eprints.nottingham.ac.uk/39211/1/Clinical%20Cancer%20research%20--revised.pdf

Copyright and reuse:

The Nottingham ePrints service makes this work by researchers of the University of Nottingham available open access under the following conditions.

This article is made available under the University of Nottingham End User licence and may be reused according to the conditions of the licence. For more details see:
http://eprints.nottingham.ac.uk/end_user_agreement.pdf

A note on versions:

The version presented here may differ from the published version or from the version of record. If you wish to cite this item you are advised to consult the publisher's version. Please see the repository url above for details on accessing the published version and note that access may require a subscription.

For more information, please contact eprints@nottingham.ac.uk

Cytoplasmic Cyclin E Predicts Recurrence in Patients with Breast Cancer

*Kelly K. Hunt, MD[1], Cansu Karakas, MD[2], Min Jin Ha, PhD[3], Anna Biernacka, MD[2], Min Yi, MD, PhD[1], Aysegul A. Sahin, MD[4], Opoku Adjapong, MD[4], Gabriel N. Hortobagyi, MD[5], Melissa Bondy, PhD[6], Patricia Thompson, PhD[7], Kwok Leung Cheung, MD[8], Ian O. Ellis, MD[8], Sarah Bacus, PhD[9], W. Fraser Symmans, MD[6], Kim-Anh Do, PhD[3], *Khandan Keyomarsi, PhD[2]

*KKH and KK contributed equally to the manuscript.

Departments of [1]Breast Surgical Oncology, [2]Experimental Radiation Oncology, [3]Biostatistics, [4]Pathology, [5]Breast Medical Oncology, and [6]Pathology Administration, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA; [6]Department of Pediatrics, Baylor College of Medicine, Houston, Texas, USA; [7]Department of Cellular and Molecular Medicine, University of Arizona Cancer Center, Tucson, Arizona, USA; [8]School of Medicine, University of Nottingham, Nottingham, UK; and [9]Quintiles Transnational Corp, Denver, Colorado, USA.

Running Title: Cytoplasmic cyclin E predicts recurrence in breast cancer

Key words: Cytoplasmic cyclin E, breast cancer, biomarker, cell cycle

Financial Support: This work was supported by the National Institutes of Health through a Cancer Center Support Grant (CA016672) to MD Anderson and grants CA87548 and CA1522218 to K. Keyomarsi, as well as through the Susan G. Komen for the Cure grant KG100521 to K.K. Hunt.

Corresponding author: Kelly K. Hunt and Khandan Keyomarsi, Department of Breast Surgical Oncology, Unit 1434, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030, USA; Phone: 713-792-7216; Fax: 713-794-5720; Email: khunt@mdanderson.org and kkeyomar@mdanderson.org.

Disclosure of potential conflicts of interest: No conflicts of interest exist.

Abstract word count: 237 **Figures and tables (main)**: 6

Manuscript word count– 5761 **Figures, Files and Tables (Supplemental):** 11

TRANSLATIONAL RELEVANCE: While molecular subtyping has helped identify distinct breast cancer subtypes, there is no biomarker yet identified that can differentiate those patients within subtypes with respect to prognosis. One of the hallmarks of cancer is the deregulation of G1 to S transition of the cell cycle. Alterations in this checkpoint can serve as an indicator of prognosis in many cancers and a potential predictor of poor outcome in breast cancer patients. We evaluated the subcellular localization of a key cell cycle regulator (cyclin E) in breast cancer specimens from 2,494 patients from 4 different cohorts from multiple institutions with distinct clinical and pathological features. In multivariable analysis we show that cytoplasmic expression of cyclin E is associated with the greatest risk of recurrence compared with other prognostic factors (including Ki67) across all subtypes and cohorts, providing a rationale for investigating treatment strategies that could specifically target tumors with cytoplasmic cyclin E.

ABSTRACT

Background: Low-molecular-weight-cyclin E (LMW-E) detected by Western blot, predicts for reduced breast cancer survival, however, it is impractical for clinical use. LMW-E lacks a nuclear localization signal which leads to accumulation in the cytoplasm that can be detected by immunohistochemistry. We tested the hypothesis that cytoplasmic staining of cyclin E can be used as a predictor of poor outcome in different subtypes of breast cancer using patient cohorts with distinct clinical and pathologic features.

Methods: We evaluated the subcellular localization of cyclin E in breast cancer specimens from 2,494 patients from 4 different cohorts: 303 from a prospective study and 2,191 from retrospective cohorts (National Cancer Institute [NCI], MD Anderson Cancer Center [MDA] and the United Kingdom [UK]). Median follow-up times were 8.0, 10.1, 13.5, and 5.7 years, respectively.

Results: Subcellular localization of cyclin E on immunohistochemistry was associated with full-length (nuclear) and low molecular weight isoforms (cytoplasmic) of cyclin E on Western blot analysis. In multivariable analysis, cytoplasmic cyclin E staining was associated with the greatest risk of recurrence compared with other prognostic factors across all subtypes in three (NCI, MDA and UK) of the cohorts. In the MDA cohort, cytoplasmic cyclin E staining outperformed Ki67 and all other variables as prognostic factors.

Conclusion: Cytoplasmic cyclin E, identifies patients with the highest likelihood of recurrence consistently across different patient cohorts and subtypes. These patients may benefit from alternative therapies targeting the oncogenic isoforms of cyclin E.

INTRODUCTION

Overexpression of low molecular weight cyclin E (LMW-E), which was originally discovered in breast cancer by our group and subsequently reported by others [1-7], is also found in ovarian cancer [8,9], melanoma [10], colorectal cancer [11-14], lung cancer [15], and renal cell carcinoma [16]. LMW-E is generated from N-terminal neutrophil elastase cleavage of the 50-KDa, full-length cyclin E1 [17]. The loss of the NH2 terminus alters the subcellular localization of LMW-E to the cytoplasm, which can no longer be degraded by the nuclear FBW-7, resulting in stable and functionally active LMW-E in the cytoplasm [18]. LMW-E is not found in normal tissues, and it causes cell cycle deregulation and resistance to endocrine therapy and chemotherapy [19-22]. Compared with full-length cyclin E, LMW-E has a higher affinity for binding cyclin-dependent kinase 2 (CDK2) and is capable of phosphorylating substrates such as pRb with higher activity [23]. Transgenic mice expressing LMW-E have a higher incidence of mammary tumor development and metastasis compared with those expressing full-length cyclin E [24], and tumorigenicity is dependent on CDK2 [25].

We previously reported an analysis of 395 patients with breast cancer in which we demonstrated that overexpression of LMW-E, as measured by Western blot analysis, is associated with distant metastases and reduced overall survival [26]. At a median follow-up of 4 years, 91.7% of patients with overexpression of cyclin E (full-length and LMW-E) according to Western blot analysis developed distant metastases, compared with 7% of patients without cyclin E overexpression ($p < 0.001$). In multivariable analysis, total cyclin E expression was an independent prognostic variable and a better predictor of both disease-free and overall survival than nodal status, estrogen receptor (ER) status, or stage.

Western blot analysis is impractical for routine clinical use, which has led several groups to examine the prognostic value of cyclin E expression measured by immunohistochemistry. In these studies, cyclin E expression was scored on the basis of nuclear staining, and no consistent association between cyclin E overexpression and reduced survival was demonstrated [27]. We previously reported that LMW-E is localized predominantly in the cytoplasm, where it binds to CDK2 and has greater kinase activity than full-length cyclin E [18]. Others have reported that mislocalization of other cyclins, including cyclins A, B, and D1, has functional implications in tumorigenesis [28-31]. These data suggest that examining tumors for expression of cyclins may be best assessed by immunohistochemistry to determine location (nuclear versus cytoplasmic) and intensity of staining. To test this hypothesis, we recently used separate nuclear and cytoplasmic scoring systems for both cyclin E and p-CDK2 expression to demonstrate altered cellular accumulation of these proteins using immunohistochemical analysis[32]. We evaluated 7 different cyclin E antibodies to identify the one antibody that can consistently detect cytoplasmic cyclin E in a panel of 14 breast cell lines and tumors from cyclin E transgenic mouse models and showed that nuclear versus cytoplasmic staining of cyclin E readily differentiated full-length from LMW-E, respectively. Lastly, we showed that cytoplasmic cyclin E correlated strongly with cytoplasmic p-CDK2 ($P < 0.0001$) in patient samples[32].

In the present study, we tested the hypothesis that expression of LMW-E, as measured by cytoplasmic staining of cyclin E in immunohistochemistry, predicts recurrence and survival outcomes in patients with breast cancer across diverse large cohorts and subtypes.

MATERIALS AND METHODS

Patients and tissue samples

Four cohorts were analyzed in the current study (Supplementary Table 1): a prospective cohort of 303 patients with stage I-III breast cancer who enrolled in our study between January 2000 and June 2010 (Lab00-222; approved by The University of Texas MD Anderson Cancer Center Institutional Review Board) and 3 retrospective cohorts, including 725 patients with stage I-II breast cancer treated at MD Anderson between 1985 and 1999 (MDA) [33], 951 patients with stage I-II breast cancer treated at four US hospitals between 1985 and 1997 (NCI) and 515 elderly patients with stage I-II breast cancer treated at Nottingham University Hospitals, Nottingham, England, between 1987 and 2005 (UK). A complete data-base of all the clinical and pathological factors evaluated for all 4 cohorts is provided in Supplemental Table 2. Fresh tumor and normal breast tissue was collected during surgery from 152 patients in the Lab00-222 cohort, and formalin-fixed, paraffin-embedded tissue was available for all patients in all four cohorts. REMARK diagram for each cohort is shown in Supplemental Figure 3.

Western blot analysis

Fresh tissues from the 152 patients in the prospective cohort were collected and protein lysates subjected to western blot using a monoclonal antibody to the C-terminus of cyclin E (HE12, Santa Cruz Biotechnology) and actin (Roche) as previously described [26,34] with the following modifications. Briefly, 50 µg of protein from each tumor tissue being examined was loaded into the lanes of a 10% SDS-PAGE gel and submitted to electrophoresis for 300 volt hours. These gels were then transferred to Immobilon P membrane (Millipore) by electrophoresis for 400 volt hours at 4°C. The membranes were blocked for 1 hour in BLOTTO (5% nonfat dry milk in TBST; 20 mM Tris, 137 mM NaCl, 0.25% Tween, pH 7.6). Following blocking, the

membranes were incubated in primary antibodies (at a concentration of 0.1 μg/mL) in BLOTTO for 3 hours at room temperature. Following incubation with primary antibody, the membranes were washed 3 x 20 minutes in TBS-T and then incubated with goat anti-mouse or anti-rabbit IgG–horseradish peroxidase-conjugated secondary antibodies (Pierce) at a dilution of 1:3,000 in BLOTTO for 1 hour. The membrane was washed 3 x 20 minutes in TBS-T and developed with the Renaissance chemiluminescence system (Perkin-Elmer Life Sciences, Inc.). The membranes were placed in an autoradiography cassette, exposed to film, and scanned. Densitometry was performed using ImageQuant Total Lab software (Amersham Biosciences), each band quantified, and LMW-E bands were added together. Values of LMW-E and full-length cyclin E were normalized against full-length cyclin E in normal tissue.

Immunohistochemistry

Slides from all patients within each cohort were stained for cyclin E using C-terminal antibody detecting full-length cyclin E and LMW-E (rabbit polyclonal antibody to cyclin E, C-19, sc-198, Santa Cruz) as recently described [35]. Briefly, Sections (5-μm-thick) of FFPE breast tumor samples were deparaffinized and rehydrated followed by incubation with 3% hydrogen peroxide and methanol to block endogenous peroxidase activity and nonspecific protein-protein interactions, respectively. Antigen retrieval was carried out with 0.01mM citric acid-based buffer at pH 6.0 using a hot plate in a metal container for 15 min before immunostaining. After 1 hour blocking for nonspecific staining, the sections were incubated with cyclin E C-19 polyclonal antibody. Antibodies were detected using a VECTASTAIN Elite ABC kit (PK6101 and PK6102; Vector Laboratories, Burlingame, CA). Cells positive for cyclin E were visualized using the chromogenic substrate 3,3'-diaminobenzidine. Slides were counterstained with hematoxylin and mounted. Tumor cell blocks known to highly express LMW-E were included in

each batch and negative control were prepared by replacing the primary antibody with PBS buffer. All washing steps were performed with, first, PBS alone and then PBS with 0.1% Tween.

Cyclin E scoring was performed by three pathologists blinded to patient outcomes. Scores (0=negative, 1=weak staining, 2=moderate staining, and 3=strong staining) were assigned for nuclear and cytoplasmic staining according to percentage of cells stained and intensity of staining (Supplemental Figure 1) and as described previously [32]. Each tumor sample was scored separately for nuclear and cytoplasmic cyclin E expression and LMW-E status was assigned as follows: LMW-E negative (no staining or just nuclear staining), LMW-E positive (nuclear + cytoplasmic or just cytoplasmic staining).

ER status, progesterone receptor (PR) status, HER2 status (3+ in immunohistochemistry or amplified in fluorescence in situ hybridization), and Ki67 status [36] were determined from pathology reports for the MD Anderson and UK cohorts. For the NCI cohort, we stained tissue samples for ER, PR, and HER2; fluorescence in situ hybridization was performed at Quintiles (Westmont, IL). Samples were collected prior to the change in American Society of Clinical Oncology/College of American Pathologists guidelines for ER reporting [37] therefore we considered >10% staining on immunohistochemistry to be positive for ER and PR.

Assignment of breast cancer subtype

ER, PR, and HER2 were used to approximate breast cancer subtypes. ER- and/or PR-positive and HER2-negative samples were considered hormone receptor-positive breast cancer; HER2-positive (independent of ER and PR status) was considered HER2-positive breast cancer; and ER-, PR-, and HER2-negative samples were considered triple-negative breast cancer (Supplementary Table 1).

Statistical analysis

Logistic regression was performed to determine whether full-length cyclin E and LMW-E expression as detected by Western blot analysis were associated with nuclear and cytoplasmic staining for cyclin E in immunohistochemistry. Differences in the cohorts were evaluated using chi-square tests for categorical variables and one-way analysis of variance for Ki67. Endpoints were overall survival and breast cancer freedom from recurrence (FFR) calculated from the time of diagnosis to recurrence. FFR is a modification from the recurrence-free survival endpoint from the Hudis et al guidelines [38] and was calculated as the time between the date of diagnosis and the date of first recurrence (locoregional or distant) or last follow-up (if no recurrence). FFR captures only recurrences and does not include deaths as events, regardless of cause of death. Patients who did not experience the endpoint were censored at last follow-up.

Median follow-up times were computed using the reverse Kaplan-Meier estimator [39]. The Kaplan-Meier method was used to calculate five-year FFR for each factor. The differences in survival curves were evaluated using log-rank test. Univariable and multivariable regression analyses were performed using Cox proportional hazards model. The final multivariable Cox models were selected using backward elimination procedure by Akaike's information criterion (AIC), which takes into account how well the model fits the data as well as the complexity of a model, thereby reducing the risk of overfitting. Model performance was quantified using concordance index (C-index) [40], which is a measure of the probability of agreement between what the model predicts and the actual observed risk of breast cancer recurrence. The variable importance in a model is evaluated using the likelihood ratio test (LRT). Statistical analyses were performed using R version 3.2.3 with library survival and rms. R markdown files described for

each cohort are presented in Supplementary R files 1-4. Each R code includes the definitions of FFR, and covariates used for each cohort, including subtype and cyclin E. The multivariable Cox-proportional hazards models for each cohort were initially fit with the following covariates: age, T stage, Nodal status, Tumor grade, Subtype, Chemotherapy, Radiation therapy, Endocrine therapy, and cyclin E. Next, backward elimination procedure was performed for variable selection. These files also include calculations of c-indices and provide codes to draw monograms.

RESULTS

Patient characteristics

There were 2,494 patients with complete data available: 303 from Lab00-222, 725 from MDA, 951 from NCI, and 515 from UK. Clinical and pathologic variables for individual cohorts and the combined cohort are summarized in Table 1. The cohorts were distinct ($P < 0.001$) with respect to all variables examined except for cytoplasmic cyclin E staining. Patients in the UK cohort were older than in other cohorts (median 76 years compared with 53-62 years; $P < 0.0001$). Additionally, T stage, nodal status, tumor grade, and subtypes were significantly different among the cohorts ($P < 0.0001$). Treatment variables (chemotherapy, radiation therapy, and endocrine therapy) were also significantly distinct among cohorts ($P < 0.0001$). In the UK cohort, not all patients received chemotherapy. These results show that although the four cohorts were distinct in all biomarkers examined, they converged with respect to cytoplasmic cyclin E staining, suggesting that cytoplasmic cyclin E may be an independent biomarker in these patients.

Levels of full-length cyclin E and LMW-E

Tumor lysates from the 152 patients from the Lab00-222 cohort were subjected to both Western blot analysis and immunohistochemistry with the cyclin E antibody. Nuclear and cytoplasmic staining of cyclin E were quantified and compared with results from the Western blot analysis. Tumors with undetectable or low levels of LMW-E in the Western blot analysis showed predominantly nuclear cyclin E staining, whereas tumors with high levels of LMW-E in the Western blot analysis demonstrated intense, homogeneous cytoplasmic cyclin E staining (Figure 1A). Logistic regression analyses were performed to determine whether LMW-E and full-length cyclin E expression in the Western blot analysis were associated with cytoplasmic and nuclear staining for cyclin E as a comprehensive score (% positive nuclei × staining intensity). Full-length cyclin E expression in Western blot analysis was an independent predictor of nuclear cyclin E staining, and LMW-E expression in Western blot analysis was an independent predictor of cytoplasmic cyclin E staining (Figure 1A). Thus, assessment of nuclear and cytoplasmic cyclin E staining in immunohistochemistry allowed separation into LMW-E (cytoplasmic) negative and LMW-E positive groups (Figures 1B, Supplemental Figures 1 and 2).

FFR and overall survival as a function of cyclin E staining

Median follow-up for the Lab00-222 cohort was 9.8 years (95% confidence interval [CI] 9.4-10.5 years), 17.9 years (95% CI 17.4-18.6 years) for the MDA cohort, 14.8 years (95% CI 14.3-15.4 years) for the NCI cohort, and 6.3 years (95% CI 6.1-7.1 years) for the UK cohort. In the combined cohort, 981 patients (39.3%) had negative cytoplasmic cyclin E staining and 1,513 (60.7%) had positive cytoplasmic cyclin E staining, and the distributions did not differ across the four cohorts (Table 1).

In the univariable Cox proportional hazards model, the association between cytoplasmic cyclin E staining and FFR was significant ($P < 0.01$) for all four cohorts (Table 2). The 5-year FFR rates for the positive cytoplasmic cyclin E staining groups compared with the negative cytoplasmic cyclin E staining groups were 86% compared with 97% for the Lab00-222 cohort, 74% compared with 93% for the MDA cohort, 69% compared with 93% for the NCI cohort, and 64% compared with 92% for the UK cohort (Supplemental Table 3). The hazard ratio for a patient with positive cytoplasmic cyclin E staining compared with a patient with negative cytoplasmic cyclin E staining was 4.16 for the Lab00-222 cohort, 3.19 for the MDA cohort, 4.16 for the NCI cohort, and 5.01 for the UK cohort (Table 2).

Kaplan-Meier FFR plots for all four cohorts and all three subtype combinations as a function of cytoplasmic cyclin E staining are shown in Figure 2. Positive cytoplasmic cyclin E staining was strongly associated with decreased FFR in all four cohorts and across all three subtype combinations, except for HER2-positive and triple-negative subtypes in the Lab00-222 cohort. The associations between cytoplasmic cyclin E staining and FFR were most striking in the hormone receptor-positive subtype.

Multivariable analysis

Clinical and treatment-related factors were represented by the following covariates: age, T stage, nodal status, tumor grade, subtype, chemotherapy, radiation therapy, and endocrine therapy. The multivariable Cox proportional hazards model was fitted in a dataset corresponding to each of the four cohorts. For these analyses, all eight clinical and treatment-related factors and cyclin E staining were initially included in the model as potential risk factors. We sequentially eliminated factors that provided decreased Akaike information criterion values to obtain the final

models. The results of the multivariable analysis are displayed in Table 3 and the corresponding nomograms are displayed in Figure 3A-D.

Cytoplasmic cyclin E staining was selected in all the four cohorts, and positive cytoplasmic cyclin E staining was strongly associated with poor outcome: FFR hazard ratios were 3.19 ($P = 0.012$) in the Lab00-222 cohort, 2.48 ($P < 0.0001$) in the MDA cohort, 3.26 ($P < 0.0001$) in the NCI cohort, and 6.58 ($P < 0.0001$) in the UK cohort. In the Lab00-222 cohort, T stage, nodal status, subtype, and chemotherapy were selected along with cytoplasmic cyclin E staining, and T stage was the most significant predictor for FFR (Table 3 and Figure 3A). In the MDA cohort, although age, T stage, nodal status, chemotherapy, and endocrine therapy were selected along with cytoplasmic cyclin E staining, the effect of cytoplasmic cyclin E staining was the strongest among these factors (Table 3 and Figure 3B). In the NCI cohort, T stage, nodal status, tumor grade, and chemotherapy were selected along with cytoplasmic cyclin E staining; cytoplasmic cyclin E staining had the strongest effect on FFR (Table 3 and Figure 3C). Lastly, in the UK cohort, age, T stage, nodal status, and endocrine therapy were included in the final model; cytoplasmic cyclin E staining had the strongest effect on FFR (Table 3 and Figure 3D). Figure 3F displays concordance indices for the final models (red) and the final models without cytoplasmic cyclin E staining for all four cohorts. The concordance indices showed substantial gains when cytoplasmic cyclin E staining was added to the model for all cohorts, especially the UK cohort, which had the largest gain.

Significance of cytoplasmic cyclin E staining from the clinical model with Ki67

Next, we sought to determine whether cyclin E had similar prognostic power using the clinical model with Ki67. Information about Ki67 was available for 692 patients in the MDA cohort.

Median Ki67 expression was 15% and ranged from 0% to 90%. According to the univariable Cox proportional hazards model, Ki67 was not significant, with a hazard ratio for a one-unit change of 1.005 (95% CI 0.998-1.013). In the multivariable analysis, the backward elimination procedure started from the model including Ki67, cytoplasmic cyclin E staining and other clinical factors, including age, T stage, nodal status, tumor grade, subtype, chemotherapy, radiation therapy, and endocrine therapy. The final model included both Ki67 and cytoplasmic cyclin E with other clinical factors, age, T stage, nodal status, chemotherapy, and endocrine therapy. Supplemental Table 4 shows the results of the final model and the corresponding nomogram is displayed in Figure 3E. The hazard ratio of positive cytoplasmic cyclin E staining compared with negative cytoplasmic cyclin E staining was 2.67 ($P < 0.0001$) and Ki67 was not significant at significance level 0.05 (Supplemental Table 4). Cytoplasmic cyclin E staining outperformed Ki67 and all other clinical factors with the highest LRT statistics with p-value <0.0001 (Supplemental Table 3). The nomogram shown in Figure 3E demonstrates that the prognostic power of cyclin E remained the same as in the model without Ki67 in Figure 3B for the MDA cohort.

DISCUSSION

In the current study, we examined the relationship between survival outcomes and LMW-E as measured by Western blot analysis and immunohistochemistry in patients with breast cancer. We found that LMW-E expression in Western blot analysis correlated with cytoplasmic cyclin E staining in immunohistochemistry. Cytoplasmic cyclin E staining was associated with reduced breast cancer FFR and overall survival in multivariable analysis in four different patient cohorts and across all breast cancer subtypes. In our study, patients with breast cancer whose

tumors had no cytoplasmic cyclin E staining had an overall favorable prognosis, and those with any cytoplasmic cyclin E staining had a poor prognosis.

A number of investigators have examined the prognostic value of cyclin E in breast cancer. A meta-analysis of 2534 patients from 12 studies demonstrated that cyclin E overexpression was associated with a 2.32-fold increased risk of recurrence in univariate analysis and a 1.72-fold increased risk of recurrence in multivariable analysis [41]. However, an attempt to validate the association between cyclin E overexpression and poor survival in patients enrolled in the Southwest Oncology Group 9313 trial using immunohistochemistry for cyclin E on formalin-fixed, paraffin-embedded tissue did not reveal significantly worse outcomes with overexpression of cyclin E [27]. We previously reported that LMW-E, not full-length cyclin E, is most active in phosphorylating substrates and that LMW-E has a higher affinity than full-length cyclin E for binding CDK [42]. LMW-E is more tumorigenic in transgenic mouse models, and patients whose tumors expressed LMW-E, as measured by Western blot analysis, were shown to be at the highest risk for recurrence and death due to breast cancer [24,26,43,44]. Because LMW-E lacks the nuclear localization signal of full-length cyclin E, we previously investigated the subcellular localization of LMW-E and reported that LMW-E accumulates in the cytoplasm, where it binds to CDK2 and retains kinase activity [18]. This difference in localization could explain why studies have shown disparate results with respect to cyclin E overexpression and survival.

Key issues in the evaluation of tumor markers for clinical use are utility, magnitude, and reproducibility [45]. The clinical utility of cyclin E as a novel tumor marker is multifaceted. In terms of reproducibility, we found that cyclin E expression was stable across multiple cohorts and subtypes of breast cancer. Hence, the expression of cytoplasmic cyclin E is not dependent on the state of differentiation of the disease, but instead is related to the aggressiveness of the

disease.

In terms of magnitude, cytoplasmic expression of cyclin E outperformed all other biomarkers examined in three of the four cohorts examined (MDA, NCI and UK), as shown in the stratified multivariate analysis with FFR as the endpoint (Table 3). To provide data for the clinical relevance of cyclin E as a prognostic marker, we interrogated if cyclin E can outperform grade and Ki67 in the ER-positive breast cancer patients. We examined grade and the expression of Ki67 when available and compared to cyclin E and the results revealed that cyclin E can separate the freedom from survival plots much more significantly than Ki67. Additionally, cyclin E assessment can be readily performed in most pathology laboratories and readily interpreted by pathologists without the need for complex algorithms required for interpretation of multi-gene assays currently in use. The 21-gene recurrence score assay (Oncotype Dx, Genomic Health, Inc.) provides prognostic information for patients with early stage, estrogen receptor positive breast cancer[46]. It also provides predictive information with respect to chemotherapy benefit in patients with a high recurrence score[47]. Another multi-gene assay is the MammaPrint test (Agendia, Inc.) which measures expression of 70 genes and categorizes tumors in patients with early stage breast cancer into low risk and high risk groups[48]. This test is similar to the 21-gene recurrence score assay in that it provides both prognostic and predictive information but is applicable to both estrogen receptor positive and negative tumor types[48]. Immunohistochemistry for localization of cyclin E provides prognostic information across all subtypes in both early stage and advanced stage breast cancer. In terms of biomarker utility, cytoplasmic cyclin E can be readily detected with immunohistochemistry. The same assay will allow detection and scoring of both nuclear and cytoplasmic cyclin E using a readily available antibody and standardized scoring. The results can be interpreted without requiring complex bioinformatics and it can

identify patients who may benefit from alternative treatment strategies targeting cyclin E.

LMW-E has greater affinity for CDK2 than does full-length cyclin E, and LMW-E is a target for CDK2 inhibitors. In HER-2-positive breast cancer cells expressing LMW-E, the combination of targeting HER-2 with trastuzumab and targeting LMW-E with roscovitine led to synergistic killing.[49] We propose that LMW-E not only is a prognostic marker but also could be useful as a predictive marker to identify patients who may benefit from LMW-E-targeted therapy. Previous studies of CDK inhibitors have not shown significant response rates, and therefore such inhibitors have been presumed to be ineffective. Measurement of cytoplasmic LMW-E may provide the best assessment of patients who could be treated effectively with LMW-E-targeted therapy. In our current study, patients with all breast cancer subtypes, including triple-negative breast cancer, had reduced recurrence-free survival when cytoplasmic cyclin E staining was detected. The lack of specific targets for triple-negative breast cancer is a major challenge, and LMW-E could be a target for this aggressive disease.

The function of cytoplasmic cyclin E goes beyond cell proliferation. Historically, the function of nuclear cyclin E has been attributed to cell cycle progression, and overexpression of nuclear cyclin E leads to deregulation of cell proliferation. Cytoplasmic cyclin E, however, has alternate functions that can affect signal transduction [50], stemness [20,51], and metabolism [52], to list a few. In fact, comparing cytoplasmic cyclin E with Ki67 revealed that cyclin E was a more significant prognostic indicator than just increased cell proliferation (Supplemental Table 4 and Figure 3E and F). The non-cell cycle functions of cytoplasmic cyclin E also provide novel avenues for targeted treatment strategies for patients whose tumors express this protein. For example, we recently identified ATP-citrate lyase (ACLY) as a novel interacting protein of LMW-E in the cytoplasm. LMW-E upregulates ACLY enzymatic activity and ACLY is required

for LMW-E mediated transformation, migration and invasion *in vitro*, as well as tumor growth *in vivo*[52]). These studies suggest a novel interplay between LMW-E and ACLY and provide an unexpected link between metabolic pathways and the cell cycle in breast cancer. Therefore, inhibition of ACLY and reduction of lipid accumulation may prove to be beneficial in targeting those breast cancers which display LMW-E expression

In summary, cytoplasmic cyclin E expression can be readily assessed at diagnosis and is an important marker of prognosis and a target for therapy in breast cancer. Our finding that LMW-E as measured by immunohistochemistry is associated with poor outcomes provides a rationale for investigating treatment strategies that could specifically target tumors with LMW-E.

ACKNOWLEDGMENTS

We are grateful to Michael Gilcrease, MD, and Thomas Buchholz, MD, for their insightful discussions during the course of this work; Dana Richardson and Tuyen Bui for technical assistance; Debra Simmons, MS, for the preparation of unstained slides; and Stephanie Deming and Erica Goodoff for editing the manuscript.

REFERENCES:

1. Mombelli S, Cochaud S, Merrouche Y, et al. IL-17A and its homologs IL-25/IL-17E recruit the c-RAF/S6 kinase pathway and the generation of pro-oncogenic LMW-E in breast cancer cells. *Sci Rep.* 2015;5:11874.
2. Tokai Y, Maeda S, Yamaguchi J, et al. Cyclin E low-molecular-weight isoform as a predictor of breast cancer in Japanese women. *Int Surg.* 2011;96(3):245-253.
3. Montazeri H, Bouzari S, Azadmanesh K, Ostad SN, Ghahremani MH. Divergent behavior of cyclin E and its low molecular weight isoforms to progesterone-induced growth inhibition in MCF-7 cells. *Adv Biomed Res.* 2015;4:16.
4. Taneja P, Maglic D, Kai F, et al. Classical and Novel Prognostic Markers for Breast Cancer and their Clinical Significance. *Clin Med Insights Oncol.* 2010;4:15-34.
5. Rath SL, Senapati S. Why are the truncated cyclin Es more effective CDK2 activators than the full-length isoforms? *Biochemistry.* 2014;53(28):4612-4624.
6. Loeb KR, Chen X. Too much cleavage of cyclin E promotes breast tumorigenesis. *PLoS Genet.* 2012;8(3):e1002623.
7. Moore JD. In the wrong place at the wrong time: does cyclin mislocalization drive oncogenic transformation? *Nat Rev Cancer.* 2013;13(3):201-208.
8. Bedrosian I, Lu KH, Verschraegen C, Keyomarsi K. Cyclin E deregulation alters the biologic properties of ovarian cancer cells. *Oncogene.* 2004;23(15):2648-2657.
9. Davidson B, Skrede M, Silins I, Shih Ie M, Trope CG, Florenes VA. Low-molecular weight forms of cyclin E differentiate ovarian carcinoma from cells of mesothelial origin and are associated with poor survival in ovarian carcinoma. *Cancer.* 2007;110(6):1264-1271.
10. Bales E, Mills L, Milam N, et al. The low molecular weight cyclin E isoforms augment angiogenesis and metastasis of human melanoma cells in vivo. *Cancer Res.* 2005;65(3):692-697.
11. Corin I, Di Giacomo MC, Lastella P, Bagnulo R, Guanti G, Simone C. Tumor-specific hyperactive low-molecular-weight cyclin E isoforms detection and characterization in non-metastatic colorectal tumors. *Cancer Biol Ther.* 2006;5(2):198-203.
12. Milne AN, Carvalho R, Jansen M, et al. Cyclin E low molecular weight isoforms occur commonly in early-onset gastric cancer and independently predict survival. *Journal of clinical pathology.* 2008;61(3):311-316.
13. Corin I, Larsson L, Bergstrom J, Gustavsson B, Derwinger K. A study of the expression of Cyclin E and its isoforms in tumor and adjacent mucosa, correlated to patient outcome in early colon cancer. *Acta Oncol.* 2010;49(1):63-69.
14. Zhou YJ, Xie YT, Gu J, Yan L, Guan GX, Liu X. Overexpression of cyclin E isoforms correlates with poor prognosis in rectal cancer. *Eur J Surg Oncol.* 2011;37(12):1078-1084.
15. Koutsami MK, Tsantoulis PK, Kouloukoussa M, et al. Centrosome abnormalities are frequently observed in non-small-cell lung cancer and are associated with aneuploidy and cyclin E overexpression. *The Journal of pathology.* 2006;209(4):512-521.
16. Nauman A, Turowska O, Poplawski P, Master A, Tanski Z, Puzianowska-Kuznicka M. Elevated cyclin E level in human clear cell renal cell carcinoma: possible causes and consequences. *Acta biochimica Polonica.* 2007;54(3):595-602.

17. Porter DC, Zhang N, Danes C, et al. Tumor-specific proteolytic processing of cyclin E generates hyperactive lower-molecular-weight forms. *Mol Cell Biol.* 2001;21(18):6254-6269.
18. Delk NA, Hunt KK, Keyomarsi K. Altered subcellular localization of tumor-specific cyclin E isoforms affects cyclin-dependent kinase 2 complex formation and proteasomal regulation. *Cancer Res.* 2009;69(7):2817-2825.
19. Nanos-Webb A, Jabbour NA, Multani AS, et al. Targeting low molecular weight cyclin E (LMW-E) in breast cancer. *Breast Cancer Res Treat.* 2012;132(2):575-588.
20. Duong MT, Akli S, Wei C, et al. LMW-E/CDK2 deregulates acinar morphogenesis, induces tumorigenesis, and associates with the activated b-Raf-ERK1/2-mTOR pathway in breast cancer patients. *PLoS Genet.* 2012;8(3):e1002538.
21. Akli S, Van Pelt CS, Bui T, Meijer L, Keyomarsi K. Cdk2 is required for breast cancer mediated by the low-molecular-weight isoform of cyclin E. *Cancer Res.* 2011;71(9):3377-3386.
22. 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(9):3198-3208.
23. Wingate H, Zhang N, McGarhen MJ, Bedrosian I, Harper JW, Keyomarsi K. The tumor specific hyperactive forms of cyclin E are resistant to inhibition by p21 and p27. *J Biol Chem.* 2005.
24. Akli S, Van Pelt CS, Bui T, et al. Overexpression of the low molecular weight cyclin E in transgenic mice induces metastatic mammary carcinomas through the disruption of the ARF-p53 pathway. *Cancer Res.* 2007;67(15):7212-7222.
25. Akli S, Van Pelt CS, Bui T, Meijer L, Keyomarsi K. Cdk2 is required for breast cancer mediated by the low-molecular-weight isoform of cyclin E. *Cancer Res.* 2011;71:3377-3386.
26. Keyomarsi K, Tucker SL, Buchholz TA, et al. Cyclin E and survival in patients with breast cancer. *N Engl J Med.* 2002;347(20):1566-1575.
27. Porter PL, Barlow WE, Yeh I-T, et al. p27Kip1 and Cyclin E Expression and Breast Cancer Survival After Treatment with Adjuvant Chemotherapy. *J Natl Cancer Inst.* 2006;98:1723-1731.
28. Moore JD. In the wrong place at the wrong time: does cyclin mislocalization drive oncogenic transformation? *Nat Rev Cancer.* 2013;13(3):201-208.
29. Diehl JA, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev.* 1998;12(22):3499-3511.
30. Faivre J, Frank-Vaillant M, Poulhe R, et al. Centrosome overduplication, increased ploidy and transformation in cells expressing endoplasmic reticulum-associated cyclin A2. *Oncogene.* 2002;21(10):1493-1500.
31. Jin P, Hardy S, Morgan DO. Nuclear localization of cyclin B1 controls mitotic entry after DNA damage. *J Cell Biol.* 1998;141(4):875-885.
32. Karakas C, Biernacka A, Bui T, et al. Cytoplasmic Cyclin E and Phospho-Cyclin-Dependent Kinase 2 Are Biomarkers of Aggressive Breast Cancer. *Am J Pathol.* 2016.
33. Thompson PA, Brewster AM, Kim-Anh D, et al. Selective genomic copy number imbalances and probability of recurrence in early-stage breast cancer. *PLoS One.* 2011;6:e23543.

34. Wingate H, Bedrosian I, Akli S, Keyomarsi K. The low molecular weight (LMW) isoforms of cyclin E deregulate the cell cycle of mammary epithelial cells. *Cell Cycle.* 2003;2(5):461-466.
35. Karakas C, Biernacka A, Bui T, et al. Cytoplasmic cyclin E and phospho-CDK2 are biomarkers of aggressive breast cancer. . *American Journal of Pathology.* 2016;In Press.
36. Cheang MC, Chia SK, Voduc D, et al. Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. *J Natl Cancer Inst.* 2009;101(10):736-750.
37. Hammond ME, Hayes DF, Dowsett M, et al. American Society of Clinical Oncology/College Of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. *J Clin Oncol.* 2010;28(16):2784-2795.
38. Hudis CA, Barlow WE, Costantino JP, et al. Proposal for standardized definitions for efficacy end points in adjuvant breast cancer trials: the STEEP system. *J Clin Oncol.* 2007;25(15):2127-2132.
39. Schemper M, Smith TL. A note on quantifying follow-up in studies of failure time. *Control Clin Trials.* 1996;17(4):343-346.
40. Harrell FE, Jr., Califf RM, Pryor DB, Lee KL, Rosati RA. Evaluating the yield of medical tests. *JAMA.* 1982;247(18):2543-2546.
41. Wang LS, ZM. Cyclin e expression and prognosis in breast cancer patients: A meta-analysis of published studies. *Cancer Invest.* 2006;24:581-587.
42. Wingate H, Puskas A, Duong M, et al. Tumor specific in breast cancer and tumorigenic in human mammary epithelial cells *Cell Cycle.* 2009;8:1062-1068.
43. Loden M, Stighall M, Nielsen NH, et al. The cyclin D1 high and cyclin E high subgroups of breast cancer: separate pathways in tumorogenesis based on pattern of genetic aberrations and inactivation of the pRb node. *Oncogene.* 2002;21(30):4680-4690.
44. Nielsen NH, Arnerlov C, Emdin SO, Landberg G. Cyclin E overexpression, a negative prognostic factor in breast cancer with strong correlation to oestrogen receptor status. *Br J Canc.* 1996;74:874-880.
45. Harris L, Fritsche H, Mennel R, et al. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. *J Clin Oncol.* 2007;25:5287-5312.
46. Paik S, Shak S, Tang G, et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. *N Engl J Med.* 2004;351(27):2817-2826.
47. Paik S, Tang G, Shak S, et al. Gene expression and benefit of chemotherapy in women with node-negative, estrogen receptor-positive breast cancer. *J Clin Oncol.* 2006;24(23):3726-3734.
48. Cardoso F, van't Veer LJ, Bogaerts J, et al. 70-Gene Signature as an Aid to Treatment Decisions in Early-Stage Breast Cancer. *N Engl J Med.* 2016;375(8):717-729.
49. Mittendorf EA, Liu Y, Tucker SL, et al. A novel interaction between HER2/neu and cyclin E in breast cancer. *Oncogene.* 2010;29:3896-3907.
50. Nanos-Webb A, Bui T, Karakas C, et al. PKCiota promotes ovarian tumor progression through deregulation of cyclin E. *Oncogene.* 2015.
51. Duong MT, Akli S, Macalou S, et al. Hbo1 is a cyclin E/CDK2 substrate that enriches breast cancer stem-like cells. *Cancer Res.* 2013;73(17):5556-5568.
52. Lucenay KS, Doostan I, Karakas C, et al. Cyclin E associates with the lipogenic enzyme ATP-citrate lyase to enable malignant growth of breast cancer cells. *Cancer Res.* 2016.

Figure Legends

Figure 1. Expression of low-molecular-weight cyclin E (LMW-E) in breast cancer. (A) Correlation between Western blot analysis and immunohistochemical analysis for LMW-E. Tumor tissues from eight breast cancer patients were examined for cyclin E expression by Western blot analysis and immunohistochemistry with an antibody targeting the C-terminal epitope of the protein. Cyclin E staining intensity and percent positivity were evaluated in both the nucleus and cytoplasm of invasive carcinoma cells and compared to the levels of full-length cyclin E and LMW-E measured by densitometry scanning of the corresponding bands on the Western blot. Invasive breast cancer with undetectable or low levels of LMW-E showed a predominantly nuclear staining pattern (a-d), whereas invasive breast cancer with high levels of LMW-E demonstrated intense, homogeneous staining throughout the cytoplasm (e-h). Table: Tumor tissues from 318 breast cancer patients from the Lab00-222 cohort were examined for cyclin E expression by western blot analysis and immunohistochemistry. Logistic regression was performed to assess if full-length and LMW-E occurrence on Western blot predict nuclear and cytoplasmic staining for cyclin E on immunohistochemistry, respectively. Statistical analysis revealed that the presence of full-length cyclin E and the presence of LMW-E on the Western blot were independent predictors of nuclear and cytoplasmic staining, respectively. LMW, low molecular weight; N, normal; T, tumor. (B) Examples of the 4 different phenotypes of cyclin E staining: no staining detected in the nucleus or cytoplasm (a), nuclear staining score exceeded cytoplasmic staining score (b), nuclear and cytoplasmic staining scores equal (c), and cytoplasmic staining score exceeding nuclear staining score (d). C, cytoplasmic; N, nuclear.

Figure 2. Kaplan-Meier survival curves demonstrating the association between cyclin E phenotype and freedom from recurrence (FFR) survival in the three subtypes, HER2 positive, hormone receptor positive, and triple negative. A. Lab222; B. MDACC TMA; C. NCI; D. UK.

Figure 3. Nomograms for 5- and 10-year freedom from recurrence survival for patients in lab222 (A), MDACC TMA (B), NCI (C), and UK (D). The nomogram that corresponds to the Cox proportional hazards model with Ki67 in the MDACC TMA cohort (E). C-indices from models with/without cytoplasmic cyclin E (F).

Table 1. Clinical, pathologic, and treatment variables for the four patient cohorts*

Variable	No. of patients (%)					P
	Combined cohort, n = 2494	Lab00-222, n = 303	MDA, n = 725	NCI, n = 951	UK, n = 515	
Age						<0.0001
Mean	61.1 yrs	57 yrs	54.1 yrs	59.6 yrs	76.2 yrs	
Median (range)	62 yrs (25-96 yrs)	57 yrs (26-92 yrs)	53 yrs (25-87 yrs)	60 yrs (25-96 yrs)	76 yrs (70-91 yrs)	
<50 years	626 (25.1)	95 (31.4)	283 (39.0)	248 (26.1)	0 (0)	
50-75 years	1346 (54.0)	180 (59.4)	383 (52.8)	577 (60.7)	205 (39.8)	
≥75 years	523 (21)	28 (9.2)	59 (8.1)	126 (13.2)	310 (60.2)	
T stage						<0.0001
T1	1282 (51.4)	164 (54.1)	396 (54.6)	557 (58.6)	165 (32.0)	
T2	1182 (47.4)	115 (38)	329 (45.4)	394 (41.4)	344 (66.8)	
T3	22 (0.9)	22 (7.3)	0 (0)	0 (0)	0 (0)	
NA	8 (0.3)	2 (0.7)	0 (0)	0 (0)	6 (1.2)	
Nodal status						<0.0001
Negative	1527 (61.2)	173 (57.1)	426 (58.8)	546 (57.4)	382 (74.2)	
Positive	952 (38.2)	130 (42.9)	298 (41.1)	391 (41.1)	133 (25.8)	
Unknown	15 (0.6)	0 (0)	1 (0.1)	14 (1.5)	0 (0)	
Tumor grade						
I	374 (15.0)	30 (9.9)	63 (8.7)	230 (24.2)	51 (9.9)	<0.0001
II	1155 (46.3)	162 (53.5)	381 (52.6)	444 (46.7)	168 (32.6)	
III	861 (34.5)	110 (36.3)	270 (37.2)	277 (29.1)	204 (39.6)	
Unknown	104 (4.2)	1 (0.3)	11 (1.5)	0 (0)	92 (17.9)	
Subtype						0.0003
HER2-positive	362 (14.5)	44 (14.5)	126 (17.4)	134 (14.1)	58 (11.3)	
Hormone receptor-positive	1640 (65.8)	217 (71.6)	451 (62.2)	660 (69.4)	312 (60.6)	
Triple-negative	428 (17.2)	39 (12.9)	148 (20.4)	140 (14.7)	101 (19.6)	
Unknown	64 (2.6)	3 (1.0)	0 (0)	17 (1.8)	44 (8.5)	
Chemotherapy						<0.0001
No	1661 (66.6)	142 (46.9)	383 (52.8)	621 (65.3)	515 (100)	
Yes	833 (33.4)	161 (53.1)	342 (47.2)	330 (34.7)	0 (0)	
Radiation therapy						<0.0001
No	1613 (64.7)	135 (44.6)	421 (58.1)	638 (67.1)	419 (81.4)	
Yes	881 (35.3)	168 (55.4)	304 (41.9)	313 (32.9)	96 (18.6)	
Endocrine therapy						<0.0001
No	1356 (54.4)	70 (23.1)	412 (56.8)	592 (62.3)	282 (54.8)	
Yes	1138 (45.6)	233 (76.9)	313 (43.2)	359 (37.7)	233 (45.2)	

Ki67						<0.0001
Mean	17.8%	12.7%	19.3%			
Median (range)	15% (0-90%)	10% (1-70%)	15% (0-90%)			
Unknown	1592 (63.8)	105 (34.7)	21 (2.9)	951 (100)	515 (100)	
Cytoplasmic cyclin E staining						0.4196
Negative	981 (39.3)	116 (38.3)	298 (41.1)	357 (37.5)	210 (40.8)	
Positive	1513 (60.7)	187 (61.7)	427 (58.9)	594 (62.5)	305 (59.2)	

*P values were calculated using one-way analysis of variance for Ki67 and chi-square tests for categorical variables.

Table 2. Univariable Cox regression analyses for freedom from recurrence (FFR) in the Lab00-222 (n = 303), MDA (n = 725), NCI (n = 951), UK (n = 515).

	Lab00-222		MDA		NCI		UK	
	HR	P	HR	P	HR	P	HR	P
Age								
<50	Ref.		Ref.		Ref.		Ref.	
50-75	0.62	0.1393	0.59	0.0004	0.74	0.0195	0.88	0.4719
>=75	0.82	0.7455	0.45	0.0282	0.68	0.0816		
T stage								
T1	Ref.		Ref.		Ref.		Ref.	
T2	1.62	0.1854	2.17	<0.0001	3.07	<0.0001	1.25	0.252
T3	9.56	<0.0001						
Nodal status								
Negative	Ref.		Ref.		Ref.		Ref.	
Positive	2.61	0.0031	1.94	<0.0001	3.16	<0.0001	1.41	0.0686
Tumor grade								
I	Ref.		Ref.		Ref.		Ref.	
II	0.98	0.9708	0.83	0.4954	1.63	0.0058	0.8	0.5425
III	2.41	0.1535	1.27	0.3651	2.76	<0.0001	1.77	0.0842
Subtype								
HER2 positive	Ref.		Ref.		Ref.		Ref.	
Hormone receptor positive	0.53	0.1079	0.61	0.0066	0.58	7.00E-04	0.64	0.1044
Triple negative	1.76	0.2083	0.94	0.7838	1.13	0.5372	1.31	0.3601
Chemotherapy								
no	Ref.		Ref.		Ref.			
yes	0.75	0.3512	1.5	0.0062	2.29	<0.0001		
Radiation therapy								
no	Ref.		Ref.		Ref.		Ref.	
yes	1.1	0.7625	1.01	0.9503	1.09	0.4917	0.9	0.6731
Endocrine therapy								
no	Ref.		Ref.		Ref.		Ref.	
yes	0.41	0.0049	0.5	<0.0001	1.15	0.2544	0.52	0.0004
Cytoplasmic cyclin E								
Negative	Ref.		Ref.		Ref.		Ref.	
Positive	**4.16**	**0.0013**	**3.19**	**<0.0001**	**4.16**	**<0.0001**	**5.01**	**<0.0001**

Table 3. Multivariable Cox regression analyses for freedom from recurrence (FFR) in the Lab00-222 (n = 303), MDA (n = 725), NCI (n = 951), UK (n = 515).

	Lab00-222		MDA		NCI		UK	
	HR	P	HR	P	HR	P	HR	P
Age								
<50			Ref.				Ref.	
50-75			0.66	0.0104			0.72	0.1303
>=75			0.48	0.0595				
T stage								
T1	Ref.		Ref.		Ref.		Ref.	
T2	0.93	0.874	1.84	<0.0001	1.84	<0.0001	1.76	0.025
T3	10.3	<0.0001						
Nodal status								
Negative	Ref.		Ref.		Ref.		Ref.	
Positive	2.15	0.045	1.88	<0.0001	2.06	<0.0001	2.33	0.0003
Tumor grade								
I					Ref.			
II					1.32	0.1518		
III					1.1.52	0.0742		
Subtype								
HER2 positive	Ref.							
Hormone receptor positive	0.43	0.056						
Triple negative	1.62	0.319						
Chemotherapy								
no	Ref.		Ref.		Ref.			
yes	0.49	0.033	0.76	0.1109	1.24	0.115		
Radiation therapy								
no								
yes								
Endocrine therapy								
no			Ref.				Ref.	
yes			0.61	0.0041			0.47	0.001
Cytoplasmic cyclin E								
Negative	Ref.		Ref.		Ref.		Ref.	
Positive	**3.19**	**0.012**	**2.48**	**<0.0001**	**3.26**	**<0.0001**	**6.58**	**<0.0001**