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TITLE: Cyclin E, A Powerful Predictor of Survival in Breast Cancer-A Prospective Study

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Aberrations in expression of cyclin E, a positive cell cycle regulator at the G1 to S transition, may affect the biological behavior of breast cancer. In a retrospective analysis of tumor specimens from 395 breast cancer patients, we found that overexpression of the full-length cyclin E protein and its LMW isoforms was a very strong predictor of breast cancer death regardless of whether patients had lymph node-negative disease or lymph node-positive disease ($p<0.0001$). Our hypothesis is that dysregulation of cyclin E is a powerful prognostic indicator of outcome in early stage breast cancer. Specifically we will: 1: use cyclin E antibody as a prognostic marker for stage I and II breast cancer in a PROSPECTIVE study, 2: examine the cyclin E associated activity and its immune-complex formation with key cell cycle regulators in freshly resected tumor samples, and 3: develop an immunohistochemical (IHC) assay for specifically detecting the LMW forms of cyclin E in Breast Cancer. We will correlate the cyclin E alterations in samples with the expression of key cell cycle regulators and clinical biomarkers. If we prospectively confirm cyclin E overexpression correlates with poor outcome, clinicians can more appropriately tailor aggressive systemic treatment to those at greatest risk for systemic metastases.
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Front Cover</td>
<td>1</td>
</tr>
<tr>
<td>SF 298 Report Documentation Page</td>
<td>2</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>3</td>
</tr>
<tr>
<td>Introduction</td>
<td>4</td>
</tr>
<tr>
<td>Body/Results</td>
<td>7</td>
</tr>
<tr>
<td>Conclusions/Discussion</td>
<td>9</td>
</tr>
<tr>
<td>References</td>
<td>10</td>
</tr>
</tbody>
</table>
Introduction:

The overall purpose of this 3 year study is to use the altered expression of cyclin E as a prognostic marker for breast cancer.

Human cyclin E was first identified in 1991 through screening of human cDNA libraries for genes that would substitute for G1 cyclin mutations in yeast [1, 2]. Further studies demonstrated that cyclin E levels were periodic during the cell cycle with levels of protein peaking in G1 [3]. This peak in cyclin E levels also correlated with maximum enzymatic function of the cyclin E-cdk2 complex [3]. The critical role of cyclin E in regulating G1 to S transition was confirmed by 2 studies in the mid-1990s. In one study, microinjection of anti-cyclin E antibodies into fibroblasts during G1 resulted in cell cycle arrest [4]. Conversely, in the other study, constitutive overexpression of cyclin E resulted in shortening of the G1 phase, decrease in cell size, and diminished requirements for growth factors [5].

Many studies point to the relevance of cyclin E alterations in breast cancer. The cyclin E gene is amplified in some breast cancer cell lines [6, 7] and we have shown that this amplification can result in as much as a 64-fold overexpression of cyclin E mRNA that is constitutively expressed across all phases of the cell cycle [8, 9]. Examination of the oncogenic potential of cyclin E in transgenic mice under the control of the bovine β-lactoglobulin promoter, revealed that lactating mammary glands of the transgenic mice overexpressing cyclin E contained regions of hyperplasia and over 10% of the mice developed mammary carcinomas [10]. Lastly, constitutive overexpression of cyclin E (but not cyclin D1 or A) in both immortalized rat embryo fibroblasts and human breast epithelial cells results in chromosomal instability [11]. Collectively, these data provide strong support for the role of cyclin E in breast tumorigenesis. We believe that the most significant cyclin E alteration is the post-translational cleavage of full-length cyclin E into low molecular weight (LMW) forms that are hyperactive compared to the full-
length protein. Some breast cancer cell lines and human breast cancers express up to 5 LMW isoforms of cyclin E (ranging in size from 34 to 49 kDa), in addition to overexpressing the 50 kDa full-length cyclin E protein [6, 12-14]. These LMW forms are unique to tumor cells and correlate with increasing stage and grade of breast cancer [12, 14-16].

Cyclin E as a Prognostic Factor in Breast Cancer

To test the clinical significance of the LMW forms of cyclin E in breast cancer prognosis, we measured expression of cyclin E in 395 women with primary breast tumors and correlated cyclin E expression with other established prognostic factors and clinical outcome. Cyclin E levels were the most powerful independent predictor for survival in stage I-III breast cancer [17]. In this study cyclin E was evaluated in breast tumors using western blot analysis. Full-length cyclin E, LMW forms, and total cyclin E levels were compared on univariate analysis with standard clinical factors (age, tumor size, nodal status, stage of disease) and biologic markers (estrogen-receptor status, progesterone-receptor status, ploidy, proliferation index, HER-2/neu status, cyclin D1 and cyclin D3). Although multiple clinical, histologic and molecular markers were significantly associated with outcome in the univariate analysis, most lost significance in the multivariate analysis. The LMW cyclin E levels and total cyclin E levels were the most powerful discriminants of overall and disease-free survival in this model and outperformed positive nodal status, stage III-IV disease status and negative estrogen-receptor status. Lymph node status has been the best prognostic factor available for assessing outcomes in women with breast cancer. In this study, cyclin E was a better prognostic indicator than nodal status and even for stage I patients who all had negative lymph nodes, cyclin E was the best indicator of outcome. These results are now being validated in a prospective trial at the M. D. Anderson Cancer Center, which is the topic of this proposal.

Cyclin E and Other Prognostic Markers in Breast Cancer
Numerous molecular markers have emerged over the past decade that may play a role in breast tumorigenesis and prognosis. These factors include estrogen-receptor, progesterone receptor, HER-2/neu and other members of the epidermal growth factor receptor family, cyclins D, E, and B, insulin-like growth factor 1 receptor, matrix metalloproteinases, p53, bcl-2, survivin, cathepsin D, and telomerase activity, to name just a few. These molecular markers frequently perform better in prognostic models than pathologic factors such as S-phase fraction, proliferation indices (Ki67) and mitotic index. The most commonly used markers in clinical practice are ER, PR and HER-2/neu. Patients whose tumors are negative for estrogen receptor have a worse prognosis than those whose tumors are positive for ER and this marker can also be used to select patients who may benefit from hormonal therapy. However, patients with estrogen receptor positive tumors still develop distant metastases and not all ER positive tumors will respond to hormonal therapy.

The human epidermal growth factor receptor 2 (HER-2/neu) is overexpressed in 25-30% of human breast cancers, suggesting a role for this overexpression in tumorigenesis [18-20]. In most cases, this overexpression is a result of gene amplification. Overexpression of this proto-oncogene in preclinical studies was associated with increased rates of cell growth, tumorigenicity and enhanced metastatic potential when transplanted into nude mice[21, 22]. Preclinical studies demonstrated that treatment of HER-2/neu overexpressing SK-BR-3 breast cancer cells with 4D5 (a murine anti-Her-2/neu antibody) inhibited cell growth [23]. A number of clinical studies have shown that breast cancers that overexpress HER-2/neu have a more aggressive course, higher relapse rate and mortality rate [19], a finding that was most pronounced in node positive patients [20, 24, 25]. Trastuzumab (Herceptin, Genentech BioOncology) was recently approved by the FDA for the treatment of HER-2/neu positive breast cancer. Trastuzumab is a humanized murine monoclonal IgG1 antibody that binds a juxtamembrane epitope in the ectodomain of HER-2/neu. Identification of HER-2/neu gene
amplification can be important in prognosis and in selecting patients for therapy with trastuzumab.

When the assessment of cyclin E was compared with both molecular markers and pathologic factors in breast tumors, all of these provided some degree of prognostic value on univariate analysis [17]. However, cyclin E was the most powerful discriminant between good and bad prognosis cohorts. When compared with the cell cycle regulators, cyclin D1 and D3, the 5-year disease-specific survival was significantly longer among patients with low levels of LMW, full-length or total cyclin E as compared with patients whose tumors had high levels of these proteins (p<0.001, log rank test). Levels (high or low) of cyclin D1 and cyclin D3 were also associated with poor disease-specific and overall survival, but less striking than those for cyclin E. Cyclin D1, cyclin D3 and HER-2/neu did not reach statistical significance when subjected to a multivariate analysis.

Results

The scope of our Statement of Work for the year 2 of the study was to continue Aim 1:

Aim 1: To use cyclin E antibody as a prognostic marker for stage I and II breast cancer in a PROSPECTIVE study (months 1-36)

A. Freshly resected breast tissue samples (normal adjacent and tumor) from 260 patients diagnosed with stage I and Stage II breast tumors will be collected; RNA, DNA and protein extracted. (months 1-24).

During the year 2 of the study we enrolled 389 patients on the study. From these 314 patients, 75 patients had neoadjuvant chemotherapy and were excluded from further analysis. Of the remaining 314 patients, 260 stage I/II patients had tissue available, where whole cell lysates were prepared from the biopsy material and subjected them to western blot analysis as follows:
0.1 gram of each matched tissue (normal adjacent tissue and breast cancer tissue obtained from the same patient) were obtained within 30-45 minutes after excision of the tumor. We extracted protein homogenates from all samples. For whole cell lysate preparation, the tissue specimen were added to one volume of sonication buffer containing a cocktail of protease and phosphatase inhibitors in a low salt buffer, minced and homogenized in a micro-mincer and sonicated at 4°C using a cup-horn adapter to eliminate probe intrusion. Homogenates were then centrifuged at 100,000 X g for 45 minutes at 4°C. The supernatants were aliquoted, and stored at -70°C and subjected to western blot analysis as described [26, 27]. The protein extracts from the 220 tumor specimen (and their corresponding normal adjacent tissue) collected thus far were subjected to Western blot analysis and the expression of cyclin E is being compared and correlated with other known prognostic markers examined in the same samples. Figure 1 depicts the summary of this study thus far:

Figure 1: Summary of tissue collection for the cyclin E study.
For each of the samples analyzed for western blot, we also performed kinase assays to examine the activity of cyclin E.

On the clinical side we obtained final IRB approval from your office on the study. The approval came on 1/18/05 and presented as figure 2.

Figure 2:
A copy of the IRB approval memo from the Army.
We have completed our goals for the first 2 years of this study which was to accrue 260 patients and subject the lysates to western blot analysis with cyclin E and other biomarkers.

Our goal for the coming year is to develop an Immunohistochemical (IHC) assay for specifically detecting the LMW forms of cyclin E in breast cancer.

References:


