Abstract. Background/Aim: Cyclin D1 is a mediator of cell-cycle control that is frequently overexpressed in primary ductal breast carcinomas, but its role is controversial. A polymorphism in the CCND1 gene, G870A, results in an aberrantly spliced protein (cyclin D1b) lacking the Thr-286 phosphorylation site necessary for nuclear export. Studies of marine fibroblasts have shown that although overexpression of canonical cyclin D1 (cyclin D1a) alone is not sufficient to drive malignant transformation, expression of nuclear cyclin D1b is oncogenic. Our objectives were to determine whether cyclin D1b is expressed in human breast carcinomas and to characterize the relationship of this protein to both cyclin D1a and clinical outcome in breast cancer patients. Patients and Methods: We performed a prospective cohort study of women with early-stage breast cancer and analyzed cyclin D1a and D1b expression in primary breast tumor sections. Expression was tested for correlation with other breast cancer prognostic factors and clinical outcome, including recurrence or death. Results: A total of 118 patients were included in this analysis, with a median follow-up of 44 months. Cyclin D1b was expressed in 26% of tumors and cyclin D1a was overexpressed in 27%; co-expression occurred in 4%. Cyclin D1a and/or D1b expression were not significantly associated with estrogen or progesterone receptor negativity, Her2 overexpression, young age, lymph node positivity, high tumor grade, nor large tumor size. The risk of recurrence was higher in those co-expressing D1a and D1b compared to the expression of either alone (relative risk=5.3, 95% confidence interval 1.27 to 22.1, p=0.02). The hazard ratio for those with co-expression compared with those without was 6.05 (p=0.04). Conclusion: Expression of cyclin D1b occurs in primary human breast carcinomas and its coexpression with cyclin D1a may be a marker for increased recurrence risk, independently of other factors.

Over 200,000 American women are diagnosed with breast cancer annually, with 40,000 deaths per year (1). Breast cancer involving axillary lymph nodes is potentially curable, but up to 50% of women with positive nodes will eventually develop metastatic disease despite optimal adjuvant treatment (2). Estrogen and progesterone receptor (ER/PR) and HER-2 status provide some predictive power for recurrence and response to targeted therapies (3). However, despite a plethora of data, few other markers clinically impact prognosis and treatment.

Cyclin D1, a protein derived from the CCND1 gene on chromosome 11q13, is involved in both normal regulation of the cell cycle and neoplasia (4-6). It is one of four cyclins that function during the G1 stage of the cell cycle by binding to cyclin-dependent kinases (CDK) 4 and 6 to promote progression to the S phase (7). The heterodimeric molecule formed by cyclin D1 and CDK 4/6 is an active protein kinase that phosphorylates specific substrates to modulate the G1/S interphase (8). After entering the S phase, phosphorylation of the cyclin D1 protein by glycogen synthase kinase-3β at a specific threonine residue (Thr-286) enables its export from the nucleus and subsequent proteolysis by Skp1-Cul1-F box4 (9, 10), thereby limiting the effect of this protein in promoting cell cycle progression (Figure 1).

A polymorphism at nucleotide 870 of the CCND1 gene, G870A, results in an aberrantly spliced cyclin D1 variant (termed cyclin D1b) (11). This mutant transcript differs from the native cyclin D1 isoform (termed cyclin D1a) in the last 55 amino acids of the carboxy-terminus, thereby lacking the Thr-286 phosphorylation site required for nuclear export and subsequent proteasome-mediated degradation (12) (Figure 2). Preliminary studies in human esophageal and breast carcinoma cell lines indicate that cyclin D1b is expressed exclusively in neoplastic cells and likely represents an oncogenic alternatively spliced isoform of canonical cyclin D1.

Key Words: Breast cancer, cyclin D1, cyclin D1b, cyclin-dependent kinase inhibitor, recurrence.
Although overexpression of cyclin D1a alone is not sufficient to drive malignant transformation in murine fibroblasts, expression of cyclin D1b, which is stabilized in the nucleus, does transform murine fibroblasts without the presence of a collaborating oncogene (13). This suggests that a polymorphism leading to the production of cyclin D1b could be the key event triggering malignancy in some patients.

We analyzed cyclin D1a and D1b expression in a cohort of women with early-stage breast carcinoma to determine the frequency of cyclin D1b expression in human breast carcinoma, determine whether it is coexpressed with cyclin D1a, and to evaluate whether cyclin D1b expression is associated with a more aggressive phenotype and a higher risk of recurrence of breast cancer.

**Patients and Methods**

We performed a prospective cohort study of patients diagnosed with early-stage breast carcinoma at the University of Pennsylvania. Patients were eligible for study participation if they had a primary tumor measuring at least 2 cm or histologically involved axillary lymph nodes, had a negative metastatic evaluation (consisting of a chest x-ray, bone scan, and serum liver function tests) and were willing to provide tumor specimens for study. At the time of enrollment, patients were required to have completed definitive surgery and lymph node evaluation with no evidence of gross or microscopic tumor at the surgical resection margins, and to have received no prior chemotherapy or radiation for the current malignancy, including pre-operative chemotherapy. Exclusion criteria included prior history of malignancy (except cervical intraepithelial neoplasia of the uterine cervix, or basal cell carcinoma), locally advanced or inflammatory breast cancer. All patients provided written informed consent prior to enrollment on the study. The study protocol was approved by the Institutional Review Board of the University of Pennsylvania and the Abramson Cancer Center Scientific Review and Monitoring Committee.
Demographic information, as well as medical, reproductive, social and family histories was collected through a questionnaire completed by each patient upon entry to the study. Information about tumor size, histological subtype, tumor grade, number of axillary lymph nodes with cancer, type of surgery, margin status, ER/PR and Her2 status were collected from each patient’s primary pathology report. Treatment information was abstracted from patient medical records. Patients were contacted on an annual basis by phone or mail to document treatment and disease status with verification via medical record review.

Immunohistochemical analysis was performed on paraffin-embedded primary tumor sections using isoform-specific antibodies to cyclin D1a and cyclin D1b by a laboratory technician blinded to patient-specific clinical data. The cyclin D1b antibody was previously described (11). Briefly, slides were sectioned at 4 to 6 microns and pretreated by microwaving in 10 mM citric acid buffer, pH 6.0, prior to treatment with 30% hydrogen peroxide to quench endogenous peroxidases. Pairs of slides from each study case were stained for cyclin D1a as well as cyclin D1b. Cyclin D1a staining was undertaken using the primary mouse anti-cyclin D1a(ab3) (Oncogene Research Products, San Diego, CA, USA) and diluted with DAKO Antibody Dilutent (DAKO, Carpenteria, California, USA) at a 1:100 dilution and incubated overnight at 4°C. A matching set of

Figure 3. Sample IHC stains of cyclin D1a and cyclin D1b. A: Cyclin D1a with strong nuclear staining (arrow) and weak cytoplasmic staining. B: Cyclin D1a with 2+ cytoplasmic staining and no nuclear staining. C: Cyclin D1b with 2+ nuclear only staining (arrow) in 100% of the cells. D: Cyclin D1b with 1+ nuclear and 1+ cytoplasmic staining in 80% of the cells.
slides was stained with primary rabbit anti-cyclin D1b (R3), produced by the Diehl lab at a 1:2,500 dilution in DAKO Antibody Diluent and incubated overnight at 4°C. Slides were then washed with 1x phosphate-buffered saline (PBS) and incubated with either Labeled Polymer HRP anti-mouse for cyclin D1a slides (K4006; Dakocytomation, DAKO, Carpinteria, California, USA) or Labeled Polymer-HRP anti-rabbit for cyclin D1b (K4003; Dakocytomation) at room temperature for thirty minutes. Slides were again rinsed with 1xPBS solution and developed with DAB+Chromogen (K3468; Dakocytomation). Slides were counterstained with Gill’s #2 Haematoxylin (Sigma-Aldrich, St. Louis, MO, USA).

Slides stained with cyclin D1a and D1b antibodies were reviewed by an independent study pathologist blinded to patient identity. The overall intensity of protein staining was recorded on a scale of 0 (no staining), 1+ (weak staining), 2+ (moderate staining), or 3+ (strong staining). Protein expression was evaluated in the nucleus and cytoplasm separately, and the percentage of cells with staining in either compartment, along with the percentage of cells with both nuclear and cytoplasmic staining, was recorded. Tumors with 2+ or 3+ staining and with greater than 50% staining for cyclin D1a in either the nucleus or cytoplasm were considered positive for cyclin D1a. Because cyclin D1b is not expected to be found to be in normal cells, tumors were considered positive for cyclin D1b if any cells showed staining in the nucleus.

Patient clinical and tumor characteristics were summarized using means, standard deviations, medians and ranges for continuous variables, and frequencies for categorical variables. Expression of cyclin D1a and D1b expression was summarized, and tested for correlation with clinical factors. Expression levels were compared between groups of patients with t-tests or their nonparametric equivalents (Wilcoxon rank sum tests); for continuous variables, Pearson and Spearman correlation coefficients were estimated. To assess the association of cyclin D1a and D1b expression with time to recurrence of disease and survival, we generated Kaplan-Meier estimates and compared these using log-rank tests. Multivariate Cox models were fit for disease-free survival (DFS) and logistic models were fit for recurrence to determine prognostic factors. Statistical analysis was performed using Stata Version 10 (College Station, TX, USA) and all tests used a two-sided significance level of 0.05.

Results
A total of 118 patients were included in this analysis, with a median follow-up of 44 months. Table I summarizes the clinical characteristics of the study population. The median age of the patients at diagnosis was 50 years, and 46% were postmenopausal. The majority of patients had positive axillary lymph nodes and high-grade tumors (79% and 60%, respectively). As expected in an early-stage cohort, the majority of tumors (80%) were hormone receptor positive, while 22% overexpressed Her2. Of the 94 patients with ER/PR-positive disease, almost all (94%) received adjuvant hormones. Most patients (76%) also received adjuvant chemotherapy with doxorubicin and cyclophosphamide.

Table I. Characteristics of study population (n=118).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Frequency</th>
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</thead>
<tbody>
<tr>
<td>Median age (years)</td>
<td>50 (range:26-84)</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>54 (46%)</td>
</tr>
<tr>
<td>Median tumor size (cm)</td>
<td>2.2 (range:1-6)</td>
</tr>
<tr>
<td>Lymph node-positive</td>
<td>93 (79%)</td>
</tr>
<tr>
<td>ER/PR-positive</td>
<td>94 (80%)</td>
</tr>
<tr>
<td>Adjuvant hormonal treatment</td>
<td>88 (94%)</td>
</tr>
<tr>
<td>No adjuvant hormonal treatment</td>
<td>2 (2%)</td>
</tr>
<tr>
<td>Adjuvant hormonal treatment unknown</td>
<td>4 (4%)</td>
</tr>
<tr>
<td>Her2-positive</td>
<td>26 (22%)</td>
</tr>
<tr>
<td>Recurrence</td>
<td>17 (14%)</td>
</tr>
<tr>
<td>Adjuvant chemotherapy</td>
<td></td>
</tr>
<tr>
<td>Doxorubicin and cyclophosphamide</td>
<td>90 (76%)</td>
</tr>
<tr>
<td>Additional taxane therapy</td>
<td>69 (58%)</td>
</tr>
</tbody>
</table>

Table II. Cyclin D1a and D1b expression patterns.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin D1a(+)</td>
<td>32 (27%)</td>
</tr>
<tr>
<td>Cyclin D1b(+)</td>
<td>31 (26%)</td>
</tr>
<tr>
<td>Nuclear expression only</td>
<td>13 (11%)</td>
</tr>
<tr>
<td>Nuclear and cytoplasmic expression</td>
<td>18 (15%)</td>
</tr>
<tr>
<td>Combination frequencies</td>
<td></td>
</tr>
<tr>
<td>D1a(+)/D1b(+)</td>
<td>5 (4%)</td>
</tr>
<tr>
<td>D1a(+)/D1b(-)</td>
<td>27 (23%)</td>
</tr>
<tr>
<td>D1a(-)/D1b(+)</td>
<td>26 (22%)</td>
</tr>
<tr>
<td>D1a(-)/D1b(-)</td>
<td>60 (51%)</td>
</tr>
</tbody>
</table>

Table III. Associations between cyclin D1a, D1b and other tumor prognostic markers.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Cyclin D1a*</th>
<th>Cyclin D1b*</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER-positive</td>
<td>25 (78%)</td>
<td>23 (74%)</td>
<td>0.57</td>
</tr>
<tr>
<td>Her2-positive</td>
<td>8 (27%)</td>
<td>5 (18%)</td>
<td>0.68</td>
</tr>
<tr>
<td>Median tumor size, cm (range)</td>
<td>1.75 (0.1-5)</td>
<td>2.5 (0.5-6)</td>
<td>0.13</td>
</tr>
<tr>
<td>Positive lymph nodes, n</td>
<td>2 (0-17)</td>
<td>1 (0-15)</td>
<td>0.21</td>
</tr>
<tr>
<td>Median age, years (range)</td>
<td>50 (38-84)</td>
<td>47 (26-81)</td>
<td>0.86</td>
</tr>
<tr>
<td>Premenopausal</td>
<td>13 (42%)</td>
<td>12 (39%)</td>
<td>0.56</td>
</tr>
</tbody>
</table>
tumors. We did not find any significant correlation between cyclin D1a and/or D1b expression and ER/PR status, Her2 status, age, lymph node status, grade of tumor, or tumor size (Table III).

At a median follow-up of 3.6 years, 17 patients (14%) had recurrence of breast cancer. Figure 4 shows the Kaplan-Meier curve for recurrence based on cyclin D1a and D1b positivity. In general, there were no significant differences in DFS comparing patients who overexpressed cyclin D1a and cyclin D1b to those who did not overexpress either isoform, or only one of the two isoforms (log-rank \( p=0.12 \)). Among patients who overexpressed either cyclin D1a or cyclin D1b, however, coexpression of cyclin D1a and cyclin D1b appeared to increase the relative risk (RR) of recurrence compared to overexpression of D1a or D1b alone (RR 5.3, 95% confidence interval (CI) 1.27 to 22.1, \( p=0.02 \)). In addition, there was a significant difference in the length of DFS according to cyclin D1a and cyclin D1b status (hazard ratio, HR=6.05, \( p=0.04 \)). When multivariate models were fit including other prognostic factors and cyclin D1a and D1b positivity, no significant predictive effects were found. The final models for both DFS and recurrence showed only tumor size as a predictive factor (\( p=0.04 \) and \( p=0.06 \) for Cox modeling of DFS and logistic modeling of recurrence, respectively).

Discussion

In this cohort of women with early-stage breast cancer, we found that 26% of tumors expressed the splice variant, cyclin D1b. The vast majority of these tumors expressed cyclin D1b in the absence of canonical cyclin D1a (4% coexpression rate), suggesting that cyclin D1a overexpression is not necessary for cyclin D1b production. Patients expressing cyclin D1b alone did not have an increased risk of relapse in our study. However, the 4% of patients with concurrent expression of cyclin D1a and cyclin D1b did show a trend toward increased risk of recurrence of breast cancer.

Cyclin D1b is expected to be found exclusively in the nucleus. We found cyclin D1b staining restricted to the nucleus in 13 breast cancers in this study; however, 18 tumors showed stainable D1b in both the cytoplasm and nucleus. These findings are consistent with studies of mantle cell lymphoma in which D1b was found in both nuclear and cytosol-protein fractions from cell lines and primary cells (14). This would seem to counter preclinical studies showing cyclin D1b restricted to the nucleus, as would be predicted, and is unexplained.

Overexpression of canonical cyclin D1a is common in breast carcinoma, with up to 50% of primary ductal breast carcinomas overexpressing cyclin D1a in various studies (15, 16). Increased expression has been shown to be evident in all histological types of breast cancer and is present in both ER\(^+\) and ER\(^-\) malignancies (17, 18). Once acquired, cyclin D1a overexpression is maintained through all stages of disease, including metastatic lesions (19). Despite the undisputed presence of cyclin D1a in breast cancer, data thus far have produced conflicting results regarding the biological and clinical significance of cyclin D1a overexpression. Cyclin D1a overexpression alone has not been shown to induce malignant transformation in cell lines and mouse models, but nuclear cyclin D1 alleles have exhibited increased oncogenic potential (20). Our data are consistent with these findings. Overall, 27% of patients overexpressed cyclin D1a and its overexpression alone did not correlate with an increased risk of relapse of breast cancer. Whether cyclin D1 overexpression is an oncogenic mechanism or simply an indication of cell proliferation driven by other upstream or downstream mechanisms remains unclear (21, 22).

Since cyclin D1b lacks the threonine-286 phosphorylation site which is required for its export out of the nucleus, it may continuously bind to CDK 4/6 to phosphorylate positive modulators of the G1/S interphase in the cell cycle. Our finding that 22% of breast tumors in our cohort expressed cyclin D1b without concurrent overexpression of cyclin D1a suggests that cyclin D1b may play a role independently of that previously attributed to canonical cyclin D1. Previous studies examining cyclin D1a expression may have erroneously measured cyclin D1b due to a lack of specificity of the antibody. It is possible that cyclin D1a may merely be a marker of increased proliferation in most patients with breast cancer, but it may represent a key driver of oncogenesis in those who express cyclin D1b as well. Thus, cyclin D1b could be the oncogenic mechanism that allows overexpression of cyclin D1a to function in an oncogenic capacity.
We are aware of only one other study testing the correlation of overexpression of the two cyclin D1 isoforms and clinical outcome in breast cancer (23, 24). These investigators studied 175 women followed for a median of 75 months, using immunohistochemical staining to identify and semi-quantitate cyclin D1a and cyclin D1b expression in formalin-fixed, paraffin-embedded tumor tissue sections, as we did (23, 24). They found a higher prevalence of both isoforms of cyclin D1 – one or both were overexpressed in 61% of tumors compared to 49% of tumors in our study. The difference was mostly in D1a, which was overexpressed in 49% of tumors they studied vs. 27% of ours; D1b was overexpressed in 31% of their tumors vs. 26% in our study. They found the D1a(+)/D1b(+) phenotype in 18% of tumors, D1a(+)/D1b(−) in 30%, and D1a(−)/D1b(+) in 12%, compared to our 4%, 23% and 22%, respectively (23, 24).

One possible reason for different staining results may be the antibodies used for detecting the D1a and D1b isoforms. Both studies used commercially available antibodies for D1a (Ab-3), although from different venders. Wang et al. used the same strategy we did to develop a similar, but not identical, antibody for detecting the D1b isoform (23). They raised antibodies to an 18-amino acid peptide derived from the C-terminal of translated intron 4 sequences that overlaps 9 peptides with the 22-amino acid sequence that we used for this purpose. Differences in the antibodies themselves or in adapting them to stain fixed, paraffin-embedded tumor tissues may account for some differences in detecting cyclin D1 overexpression.

Millar et al. (24) found that D1b overexpression was significantly associated with poor outcome in the form of shorter time to recurrence, distant metastasis and breast cancer-specific death; patients with the D1a(+)/D1b(+) and D1a(−)/D1b(+) had the worst outcomes (24). In contrast, we saw poorer outcome only in patients with the D1a(+)/D1b(+) phenotype. Like us, they did not find any significant associations between D1b expression and tumor size, lymph node status, hormone receptor expression or Her2 status, nor did they find an association between high cyclin D1a expression and outcome (24).

The lack of a greater prognostic effect of cyclin D1b in our cohort may be due to several factors. Clearly, the sample size and small number of relapses at the time of this analysis may have limited our ability to detect a significant difference. Notably, after a median of 44 months of follow-up, only 14% of our patients had relapse of their breast cancer, suggesting that these patients are doing well in the short term with improvements in therapy, especially dose-dense treatment in patient with large tumors or with lymph node-positive disease and the increased use of aromatase inhibitors in postmenopausal women since the start of the study. Addressing the possible effects of treatment, we note in Millar et al., only 41% of their patients received adjuvant chemotherapy vs. 76% of our patients. Similarly, 51% of their patients received adjuvant endocrine therapy vs. 94% in our group (24). Longer follow-up may provide greater evidence of a correlation between cyclin D1a overexpression, cyclin D1b expression, and risk of relapse of breast cancer in our cohort.

In conclusion, our study shows that cyclin D1b is found in human breast carcinoma and that cyclin D1a overexpression or cyclin D1b expression alone does not correlate with any adverse prognostic markers or with clinical outcome. However, coexpression of cyclin D1a and D1b proteins may have an implication for risk of recurrence. The recent development of CDK inhibitors targeting the cyclin D1 pathway lends special importance to exploring this pathway and identifying patient populations who would benefit most from these drugs. Identifying patients in whom cyclin D1a or D1b overexpression is a key activator of oncogenesis is crucial in selecting patients rationally for future trials of drugs targeting cyclin D1 modulators. These findings suggest that cyclin D1b warrants further investigation as an oncogenic marker in breast cancer.

Acknowledgements

This study was made possible through generous funding from the NCI (Angela DeMichele: K23-CA81009; Alan Diehl: CA 11360; Vandana Gupta Abramsen: 5-K12-CA-076931-10), and through the Leukemia Lymphoma Society (Alan Diehl: Leukemia Lymphoma Society Scholar).

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Received December 4, 2009
Revised March 19, 2010
Accepted March 22, 2010