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Cyclin D1 and Estrogen Receptor Messenger RNA Levels Are Positively Correlated in Primary Breast Cancer


Cancer Biology Division, Garvan Institute of Medical Research, St. Vincent’s Hospital, 384 Victoria Street, Darlinghurst, Sydney, New South Wales 2010, Australia [R. H., A. L. C., E. A. M., R. L. S.]; The Breast Cancer Unit, Tenovus Cancer Research Centre, University of Wales College of Medicine, The Heath, Cardiff, CF4 4XX, United Kingdom [R. A. M., R. I. N.]; and Department of Surgery, City Hospital, Hucknall Road, Nottingham, NG5 1PB United Kingdom [J. F., R. R., R. W. B.]

Abstract

The CCND1 gene, encoding the cell cycle regulatory protein cyclin D1, maps to chromosome 11q13, a locus that is amplified in about 13% of breast cancers. Because several studies have indicated a relationship between 11q13 amplification and markers of phenotype including estrogen receptor (ER) status, we tested the relationship between CCND1 and ER gene expression in 364 primary breast cancers using Northern blot analysis. Seventy-three percent of samples were positive for ER mRNA, and cyclin D1 mRNA levels in the ER-positive group were significantly greater than those in ER-negative samples (P = 0.0001). When the samples were divided into quartiles of cyclin D1 expression, 58% of samples were positive for ER mRNA, and cyclin D1 mRNA levels in the ER-positive quartile were significantly higher than those in the ER-negative quartile (P = 0.0001). Using simple regression analysis, there was a significant positive correlation between cyclin D1 mRNA and ER mRNA levels in the total population (P = 0.0001). This study demonstrates that cyclin D1 mRNA and ER mRNA are positively correlated in primary breast cancer, but the functional relationship between these genes remains to be elucidated.

Introduction

Cyclin D1 plays a pivotal role in regulating the progression of diverse cell types through the G1 phase of the cell cycle (1). Recent data also indicate a critical role for cyclin D1 in normal mammary gland development. Cyclin D1 knockout mice fail to develop lobular alveoli during pregnancy (2, 3), whereas transgenic mice expressing this gene in the mammary gland have premature lobular alveolar development and develop adenocarcinoma, albeit after a long latency period (4). In human breast cancer cells, induction of cyclin D1 is an early response to mitogenic stimulation by growth factors and steroids (5), whereas antiestrogen inhibition of breast cancer cell proliferation is preceded by decreased cyclin D1 mRNA and protein expression (5, 6). Furthermore, in T-47D cells expressing cyclin D1 under the control of an inducible promoter, induction of cyclin D1 is sufficient for serum-deprived, growth-arrested cells to resume cell cycle progression and subsequently complete DNA synthesis and cell division (7). This suggests that deregulated expression of cyclin D1 provides a growth advantage to tumor cells, perhaps by reducing dependence on normal physiological growth stimuli. Thus, cyclin D1 is a putative oncogene, but its role, if any, in the development and progression of human breast cancer remains to be defined.

Much of our current knowledge on the role of cyclin D1 in breast cancer has been deduced from the fact that the CCND1 gene encoding cyclin D1 is located at chromosome 11q13. Several studies have reported a positive correlation between 11q13 amplification and ER positivity (8–12), whereas other groups did not find such an association (13–17). A pilot study performed in this laboratory in a series of 124 primary breast tumors and 16 histologically normal breast tissue samples indicated that 45% of breast cancers expressed high levels of cyclin D1 mRNA (18), 3-fold higher than predicted from the average frequency of 11q13 amplification of 13.3% (19). We also noted that tumors with the highest levels of cyclin D1 mRNA expression were uniformly positive for ER (18). Four more recent studies, which examined the expression of cyclin D1 protein in primary breast cancer by immunohistochemical staining, confirmed that the frequency of cyclin D1 abnormalities is much higher than suggested previously from data on DNA amplification (20–23). Thus, cyclin D1 overexpression can result from mechanisms other than gene amplification, as documented previously in breast cancer cell lines (18). These data highlight the need to reassess the relationship between cyclin D1 mRNA expression and clinicopathological parameters, given previous suggestions of a relationship between 11q13 amplification and ER/progesterone receptor status, lymph node involvement, and survival (8, 10, 11, 14). The present study addresses further the relationship between cyclin D1 expression and phenotype in primary breast cancer by establishing a positive relationship

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2 To whom requests for reprints should be addressed. Phone: 61-2-295-8322; Fax: 61-2-295-8321.


4 The abbreviation used is: ER, estrogen receptor.
Table 1  The relative expression of cyclin D1 mRNA in the four control cell lines employed as standards on all 22 filters used in the study.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>HBL-100</th>
<th>MDA-MB-231</th>
<th>MCF-7M</th>
<th>MDA-MB-134</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SE</td>
<td>0.229 ± 0.015</td>
<td>1.0</td>
<td>3.628 ± 0.244</td>
<td>7.903 ± 0.665</td>
</tr>
<tr>
<td>Variance</td>
<td>6.6%</td>
<td>6.7%</td>
<td>8.4%</td>
<td></td>
</tr>
</tbody>
</table>

between cyclin D1 and ER mRNA expression in a large series of 364 breast carcinomas.

Materials and Methods

Tumor Samples and Control Cell Lines.  The breast cancer samples were collected at surgery within the Department of Surgery, City Hospital, Nottingham, United Kingdom. Samples of tumor tissue were rapidly frozen, stored at −70°C, and transported on dry ice to be processed in the Breast Cancer Unit, Tenovus Cancer Research Centre, Cardiff, United Kingdom. RNA was extracted from the samples as described below and transported subsequently on dry ice to the Cancer Biology Division, Garvan Institute of Medical Research, Sydney, Australia for further analyses. RNA from the breast cancer cell lines MDA-MB-134, MCF-7M, MDA-MB-231, and HBL-100, which show a wide range of cyclin D1 expression (18), were included on each blot as positive and negative controls. HBL-100 and MDA-MB-231 are ER negative, whereas MCF-7M and MDA-MB-134 are ER positive. MDA-MB-134 was obtained from the American Type Culture Collection (Rockville, MD), and MCF-7M, MDA-MB-231, and HBL-100 cells were obtained from E. G. and O. Mason Research Institute (Worcester, MA). Cell lines were cultured, passaged, and harvested for total RNA preparation as described previously (18).

cDNA Probes.  The human cyclin D1 cDNA, a 1.3-kb HindIII restriction fragment encompassing the whole open reading frame of cyclin D1 and portions of untranslated 5’ and 3’ regions, was provided by Drs. Yue Xiong and David Beach of the Cold Spring Harbor Laboratory (Cold Spring Harbor, NY). The ER cDNA was a 2.1-kb EcoRI fragment of the human ER αO8 clone, which encodes the open reading frame and a portion of the untranslated 3’ region.

RNA Isolation and Analysis.  Total RNA was isolated from primary breast cancers using the guanidinium isothiocyanate-cesium chloride method as described previously (18). Total tumor RNA (10–20 μg) was electrophoresed through a 1% agarose gel containing 2.2 M formaldehyde, 40 mM morpholinoopranesulfonic acid (pH 7.0), 10 mM sodium acetate, 1 mM EDTA (pH 8.0) and ethidium bromide, and then transferred onto Zetaprobe nylon membranes (Bio-Rad, Hercules, CA). The RNA was fixed to the membranes using UV irradiation. Filters were probed with [α-32P]dCTP-labeled cyclin D1 cDNA in the presence of 50% (v/v) deionized formamide, 2× SSPE (1× SSPE = 0.15 M NaCl, 10 mM NaH2PO4, 1 mM EDTA, pH 7.4), 1% SDS, 0.5% Blotto (10% skim milk powder in 0.2% azide), 10% dextran sulfate (molecular weight, 500,000), 0.04 mg/ml polyadenylic acid (5’), and 0.5 mg/ml salmon sperm DNA, at 50°C. After hybridization, the filters were washed at a highest stringency of 0.2× SSC (1× SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.0) and 1% SDS for 30 min at 65°C. The filters were then exposed to X-ray films and PhosphorImager screens. Differences in hybridization intensity were measured by Molecular Dynamics PhosphorImager Scanner (model 445 SI) and analyzed by Signal Analytics Corporation’s IPLab Gel software program. Cyclin D1 cDNA was stripped by heating to 100°C for 5 min in a solution of 0.1× sodium chloride/sodium citrate buffer and 0.1% SDS. The filters were subsequently probed with [α-32P]dCTP-labeled ER cDNA and quantitated as described above.

Statistical Analysis.  The data were analyzed using the software program StatView SE + Graphics (Abacus Concepts, Inc.). The correlation between cyclin D1 and ER mRNA levels was determined using simple regression analysis and the non-parametric Mann-Whitney U test.

Results

The 364 RNA samples were analyzed on 22 separate filters containing up to 18 breast cancer samples per filter and a lane for each of the four control cell lines HBL-100, MDA-MB-231, MCF-7M, and MDA-MB-134. Preliminary experiments employing 2-fold serial dilutions of RNA from each of the four control cell lines established that cyclin D1 expression could be measured over an approximately 30-fold range (Table 1) by loading 20 μg of total RNA from HBL-100 and MCF-7M, 10 μg of MDA-MB-231, and 5 μg of MDA-MB-134, which over-expresses cyclin D1 because of an approximate 14-fold amplification of the CCND1 gene. A representative filter probed sequentially with cyclin D1 and ER cDNAs is illustrated in Fig. 1, where it can be seen that the 4.5- and 1.5-kb cyclin D1 transcripts reported previously in various tissues, including breast cancer cell lines, were defined clearly in the tumor RNA. Similarly, the predominant 6.3-kb ER transcript was defined clearly in RNA from both cell lines and tumor samples. To enable comparison between filters, the cyclin D1 mRNA signal intensity of each individual tumor sample was normalized to that of MDA-MB-231, which was defined arbitrarily as 1.0 to yield “relative expression of cyclin D1.” Similarly, the relative expression of ER mRNA was obtained by normalizing the signal intensity to that of MCF-7M. The variability between filters was small, as evidenced by the relative cyclin D1 mRNA expression of each cell line having a variance of less than 10% in all cases (Table 1). Although there was little variation in RNA loading, as evidenced by ethidium bromide staining of the gel before transfer (Fig. 1), two independent observers considered that 49 RNA samples were degraded based on the integrity of the 28S and 18S rRNA subunits.

All samples expressed detectable cyclin D1 mRNA. Cyclin D1 showed a unimodal frequency distribution (Fig. 2), and the relative cyclin D1 mRNA levels of the tumor samples ranged up to 20.19, with a median of 1.04. In contrast, some samples did
Expression of cyclin D1 and ER mRNA in 18 samples of primary breast cancer. A representative filter containing the four control cell lines HBL-100, MDA-MB-231, MCF-7M, and MDA-MB-134 (20, 10, 20, and 5 μg of total RNA, respectively) and 20 μg RNA from each of 18 breast cancer samples was probed sequentially with cyclin D1 and ER cDNA. RNA loading was assessed by ethidium bromide staining. The samples in Lanes 12 and 13 (center of figure) are degraded. ●, cyclin D1 and ER mRNA species; ●, 18S and 28S rRNA.

not express detectable ER mRNA when maximal contrast was applied to the images of the gels from the Phosphor-Imager and were therefore judged to be ER negative. Two hundred sixty-four (73%) of 364 samples were ER positive. The relative ER mRNA expression ranged from 0 to 17.18; the median was 1.02. The frequency distribution of the ER-positive group was unimodal (Fig. 2).

To establish an initial relationship between cyclin D1 mRNA levels and ER status, ER positivity in different quartiles of cyclin D1 expression was calculated. The number of ER-positive samples increased with ascending cyclin D1 quartiles from 58% in the lowest quartile to 87% in the highest quartile (Fig. 3). Only three ER-negative tumors were identified among the 10% of tumors expressing the highest cyclin D1 levels.

We next determined quantitative relationships between cyclin D1 and ER mRNA. The relative ER expression in the 50% of samples with lower cyclin D1 mRNA levels was significantly less than that in the 50% with higher cyclin D1 expression (P = 0.0001). Similarly, the relative cyclin D1 mRNA expression in the ER-positive group was significantly higher than that in the ER-negative group (P = 0.0001). Finally, using simple linear regression analysis on the total population of 364 samples, there was a significant positive correlation between the relative expression of cyclin D1 and ER mRNA expression (P = 0.0001; Fig. 4). The significance of the relationship did not change when the 49 apparently degraded samples were excluded from the analysis.

Discussion

This study has documented the levels of cyclin D1 and ER mRNA in a large series of 364 primary breast cancers and demonstrated a significant positive correlation between the expression of these two genes. It thus confirms the positive correlation between 11q13 amplification and ER status noted in some earlier studies (8–12). Furthermore, the demonstration of a significant relationship between the levels of expression of these two genes, which are thought to be intimately involved in the development and progression of breast cancer, raises major issues regarding their functional interrelationships and consequently their potential role as prognostic factors, response parameters, and therapeutic targets.

ER gene expression has been studied widely in breast cancer using a range of methods including radioligand binding, enzyme immunoassay, immunohistochemistry, Northern blotting, and reverse transcriptase-PCR (24–26). This is the largest study using Northern analysis, and the data are in general agreement with other more conventional methods (27). In our study, 73% of samples expressed detectable ER mRNA, and this is consistent with previous assessments of ER status in similar series of patients (28, 29).

The ER frequency distribution curve shown in Fig. 2A is skewed markedly and similar to that observed using radioligand binding (30, 31). A bimodal distribution was observed previously when ER was quantitated by enzyme immunoassay (32, 33), but this was not evident in our study. Significantly greater
numbers of samples would need to be assessed, however, before such a relationship could be accurately determined at the mRNA level.

The frequency distribution of cyclin D1 also demonstrated a wide range of mRNA levels (Fig. 2B), but, unlike the ER frequency distribution, there was no clear cutoff to suggest a significant population of cyclin D1-negative samples. This suggests that cyclin D1 may be essential for breast epithelial cell viability, a view supported by the fact that cyclin D1 is essential for normal development of mammary gland (2, 3). Although it has been established that cells with dysfunctional retinoblastoma protein have decreased cyclin D1 expression (e.g., Ref. 21), the frequency distribution in Fig. 2B failed to identify a distinct population of low cyclin D1 expressers, which potentially might identify tumors with dysfunctional retinoblastoma protein. Buckle y et al. (18) also reported a similar cyclin D1 mRNA frequency distribution in 124 primary breast cancer samples and noted that 45% of samples expressed higher levels of cyclin D1 as compared to the 16 normal breast tissue samples. The proportion of cyclin D1 overexpressers in that study is consistent with more recent data from immunohistochemical analysis (20–22). Interestingly, Bartkova et al. (21, 22) also noted that cyclin D1 expression was similar in ductal carcinoma in situ and invasive breast cancers, suggesting that these molecular changes may occur relatively early in tumorigenesis. In the present study, we did not define the percentage of tumors overexpressing cyclin D1 due to CCND1 gene amplification, but paired DNA samples are available, and this relationship will be established subsequently.

On reviewing the published literature, five studies reported a strong association between ER positivity and amplification of the 11q13 locus, where the CCND1 gene is located (8–12). Two studies noted a trend for this correlation (34, 35), whereas five others found no association (13–17). Because the two largest series with more than 1000 samples each showed a positive correlation (11, 12), the relationship with ER status appears to be the strongest association between 11q13 amplification and any known prognostic indicator. There are limited published data on the relationship between cyclin D1 and ER expression. McIntosh et al. (23) failed to find an association between cyclin D1 and ER expression using immunohistochemistry in 93 breast cancer samples. On the contrary, Buckley et al. (18) noted that tumors expressing the highest levels of cyclin D1 mRNA were all ER positive. The present study establishes clearly a relationship between cyclin D1 and ER status and expression, and this supports earlier conclusions from studies based on 11q13.

Fig. 3 Relationship between ER status and cyclin D1 mRNA levels. Data are presented as the percentage of ER-positive (▲) and ER-negative (■) primary breast cancer samples in each quartile of relative cyclin D1 mRNA expression.
amplification. However, mRNA and protein expression may diverge especially for gene products where posttranscriptional mechanisms are important in regulation. Consequently, a detailed analysis of the relationship between cyclin D1 and ER protein may shed more light on this relationship.

Some studies also suggested that 11q13 amplification, and therefore cyclin D1 overexpression, predicts an increased risk of relapse (10, 14, 16, 35, 36). On the other hand, two larger studies, including our own, did not show such an association for all patients (12, 17), but trends were evident in the ER-positive and lymph node-negative good-prognosis subgroups (12). The true prognostic significance of cyclin D1, therefore, remains to be defined by future studies based on measurement of the gene product. Borg et al. (10) noted that relapse-free survival was shorter in the subgroup with 11q13 amplification and positive ER. If cyclin D1 is a marker of poor prognosis, then overexpression of cyclin D1 and ER may identify a subset of poor prognosis patients within the ER-positive, good-prognosis group. Such a subset of patients was identified in our recent study on 11q13 amplification (12), and these may benefit from more aggressive treatment. Unfortunately, there is insufficient follow-up at this time to address this issue in the present series.

Many breast cancers are dependent on estrogen for growth, and there is increasing evidence that estrogen is an important underlying factor in the development and maintenance of breast cancer. Estrogen treatment of growth-arrested MCF-7 cells has been shown recently to increase the expression of cyclin D1, and this is associated with cyclin D1/CDK4 complex formation and an increase in cell cycle progression. Thus, tumors with functional ER may synthesize more cyclin D1, which is potentially an underlying mechanism for the positive correlation between cyclin D1 and ER expression. If this was the case, expression of cyclin D1 in ER-positive tumors might be predicted to more accurately identify the hormone-responsive phenotype, as is the situation in ER-positive/progesterone receptor-positive tumors. Conversely, overexpression of cyclin D1 in ER-positive tumors may confer insensitivity to some therapies, because decreased cyclin D1 expression is an early event following antiestrogen treatment (6), and ectopic expression of cyclin D1 can induce resumption of cell cycle progression in antiestrogen-arrested T-47D cells. Therefore, an important unanswered question is the functional consequences of cyclin D1 overexpression, particularly in relation to sensitivity to endocrine therapy. This issue is being addressed currently in vitro and in tumor samples from patients with known responsiveness to endocrine therapy.

In conclusion, the present study establishes a significant positive correlation between cyclin D1 and ER mRNA expression, and suggests some potential clinical implications of high cyclin D1 expression. Assessment of the relationship between cyclin D1 overexpression and survival is not included in the present study, in view of the relatively short follow-up in this cohort of patients. However, this important relationship will be investigated in subsequent studies.

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References


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