Cyclin D1 Protein Is Overexpressed in Hyperplasia and Intraductal Carcinoma of the Breast

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INTRODUCTION

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ABSTRACT

The cell cycle regulatory gene cyclin D1 is a candidate oncogene in breast cancer. It is overexpressed in 30–50% of invasive primary breast cancers and plays a key role in mediating mitogenic responses to steroids and growth factors in breast cancer cells in vitro. Because the role of cyclin D1 in the proliferative and early noninvasive stages of breast cancer is largely unknown, we examined normal breast epithelium (NBE), proliferative disease (PD), ductal carcinoma in situ (DCIS), and invasive carcinoma (IC) to evaluate the timing and possible importance of cyclin D1 expression in the development of breast cancer. Using immunohistochemistry, we examined cyclin D1 protein expression in 471 breast tissue samples. A quantitative scoring system for immunohistochemistry based on percentage of positive cells was developed that correlated with Western blot analysis of antigen concentration in paired samples ($r^2 = 0.91, P = 0.003$). A sample was considered positive if $>5\%$ of relevant epithelial cells demonstrated nuclear staining. Cyclin D1 positive samples were observed in 11.7% (7 of 60) of NBE, 25% (11 of 44) of PD without atypia, 39.4% (13 of 33) of atypical ductal hyperplasia, 43.6% (17 of 39) of low-grade DCIS, 47.9% (23 of 48) of high-grade DCIS, and 48.3% (99 of 205) of IC. Cyclin D1 expression was significantly higher in PD than NBE ($P = 0.006$) and in DCIS than PD ($P = 0.038$). There was no significant increase from DCIS to IC ($P = 0.52$). The increase in cyclin D1 expression in the overall progression from NBE to IC was also highly significant ($P = 0.0001$). Therefore, cyclin D1 expression was detected at levels significantly greater in PD than NBE in the earliest proliferative epithelial lesions of the breast with a further significant increase accompanying the progression to any form of cancer. This suggests that overexpression of cyclin D1 protein is important at the earliest stages of breast oncogenesis and continues to have a crucial role throughout the development of malignancy.

INTRODUCTION

Although the exact sequence of events in the development of breast cancer has not been established, the histological model of progression from NBE to PDWA, ADH, DCIS, and IC may provide a paradigm for examination of genetic changes associated with the development of malignancy (1, 2). Using this model, it is possible to examine and compare genetic alterations and changes in gene expression between different histological groups and to make inferences about the role of known and putative oncogenes and tumor suppressor genes. Examination of chromosomal abnormalities has demonstrated shared loss of heterozygosity patterns in DCIS and IC in the same breast: at 2pter, 16q21 (2); at 8p, 13q, 16q, and 17p; and in the FHIT and ATM genes; and shared regions of amplification on chromosomes 3, 10, 17, 19, and 20 (3–7). Gene expression studies have shown similar levels of expression of p53, c-erbB-2, and ER in DCIS and IC (8–10). Although some changes, such as loss of heterozygosity in the FHIT and PTEN genes and alterations in p53 expression, have been demonstrated in PD (2, 11), other chromosomal abnormalities and aberrant gene expression in these lesions appear to be less frequent (9, 12).

The cell cycle regulatory gene, cyclin D1, is emerging as a potentially significant oncogene in invasive breast cancer. Cyclin D1 functions as a key regulator of progression through the $G_1$ phase of the cell cycle (13). In human breast cancer cells, cyclin D1 expression is increased in response to mitogenic steroids and growth factors and decreased preceding antisterogen inhibition of cell proliferation (14). Ectopic expression of cyclin D1 is sufficient to initiate cell cycle progression in the absence of external growth stimuli (15). Studies in mice have demonstrated that cyclin D1 plays a pivotal role in normal mammary gland development. Cyclin D1 knockout mice fail to undergo the lobular proliferative changes normally seen in the breast epithelial compartment under the influence of steroid hormones in pregnancy (16), whereas overexpression of cyclin D1 in the mammary gland of transgenic animals results in premature lobulo-alveolar development, abnormal epithelial proliferation in pregnancy, and the late development of adenocarcinoma (17). Evidence of functional links between steroid hormone action and cyclin D1 expression implicit in these animal studies and in the similarity in phenotype between cyclin...
D1 and progesterin receptor null mice (18) is strengthened by recent data showing that cyclin D1 and ER gene expression are positively correlated in primary breast cancer (19, 20) and that estrogen stimulation increases cyclin D1 gene expression in an ER-positive cultured breast cancer cell line (21).

Therefore, with increasing understanding of the action of cyclin D1 in cell cycle control, its role in murine mammary gland development and oncogenesis, and apparent association with estrogen and progesterin action, it is possible to hypothesize that the overexpression of cyclin D1 may contribute to a loss of normal growth control, characteristic of the early stages of breast cancer development in humans. Protein and mRNA overexpression of cyclin D1 have been demonstrated in ~50% of primary breast cancers (20, 22–28). Correlation of protein overexpression was seen in DCIS and IC in 98% of 96 specimens examined by IHC (23), and mRNA overexpression was detected by ISH in 87% of comedo and 76% of noncomedo DCIS (29). In contrast, quantitatively increased mRNA expression was observed in only 18% of 22 PD lesions in the same study.

Cyclin D1 protein expression in PD and early DCIS has not been examined, possibly because assessment of gene expression in these lesions is limited by the need to preserve histological details to confirm the diagnosis. Microdissection and ISH techniques are presently suitable only for small numbers. IHC allows examination of a large series of cases while preserving histological features. However, quantitation of cyclin D1 expression by IHC has not been standardized, with some series reporting assessment of numbers of positive cells (25–27, 30, 31) and others intensity of nuclear staining in individual nuclei (23, 28), a feature that can be reliably assessed for some proteins by computerized image analysis of optical or color density (32–34). Thus, the aim of this study was to examine, using IHC and a validated quantification assessment technique, a series of breast samples of NBE, PD, DCIS, and IC to determine the timing and extent of cyclin D1 protein overexpression in a model of human breast cancer development.

MATERIALS AND METHODS

Breast Tissue Samples. Formalin-fixed, paraffin-embedded samples of operative breast tissue specimens from reduction mammoplasties, biopsies that yielded benign disease, and cancer procedures from July 1991 to June 1996 were collected retrospectively from the Department of Anatomical Pathology archives at St. Vincent's Hospital, Sydney, Australia. Blocks were stored at room temperature, and sections were cut as required less than 1 week before staining.

Immunohistochemistry. Sections (3 μm thick) of each block were mounted on SuperFrost/Plus slides (Menzel-Glaser), air-dried overnight, and heated to 60°C for 1 h to promote section adherence. Cut sections were stored at 4°C. Prior to staining, the sections were dried overnight at 37°C, deparaffinized in xylene, and rehydrated in an alcohol series (100, 70, and 40%). Antigen unmasking was accomplished using a high temperature technique by boiling under pressure in a 0.1 M citrate buffer (pH 6.0) at 116°C for 2 min then cooling for 20 min in a water bath (20, 35). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide at room temperature for 10 min, followed by two PBS washes (pH 7.4). Nonspecific epitopes were blocked with 5% normal horse serum in PBS (room temperature for 20 min, no PBS wash). Sections were then incubated with 1:50 anti-cyclin D1 (NCL-Cyclin D1; Novocraft Laboratories, Ltd., Newcastle-upon-Tyne, United Kingdom) in 2% BSA/PBS (4°C overnight). After two PBS washes, primary antibody binding was detected with 1:200 biotinylated horse anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA; 30 min at room temperature) and an avidin-biotin-peroxidase and diaminobenzidine color detection system used according to manufacturer's instructions (Vectorstain Elite ABC and diaminobenzidine kits; Vector Laboratories). The monoclonal antibody to cyclin D1 demonstrated no cross-reactivity with cyclins D2 or D3 using immunoblotting techniques (36). Sections were lightly counterstained with Harris hematoxylin.

Cell Lines and Culture. HBL-100, MDA-MB-231, and T-47D cell lines were obtained from EG and G Mason Research Institute (Worcester, MA). MCF-7 cells were obtained from the Michigan Cancer Foundation (Detroit, MI); MDA-MB-134 cells were from the American Type Culture Collection (Rockville, MD); and HMEC-184 cells were supplied by D. Martha Stapper (University of California, Berkeley, CA). All lines except HMEC-184 were grown in RPMI 1640 supplemented with 6 mM L-glutamine, 14 mM sodium bicarbonate, 20 mM HEPES, 10 μg/ml human insulin (CSL-Novo, North Rocks, New South Wales, Australia), and 10% FCS. HMEC-184 cells were maintained in MEGM medium with 0.4% bovine pituitary extract from Clonetics Corp. (San Diego, CA).

Cell Blocks. Cultured, exponentially growing cells were harvested with trypsin, washed in PBS, centrifuged for 10 min at 1000 rpm to form a pellet, and resuspended in a clot of human serum. The cell suspension was fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin in the same manner as the tissue samples to form a cell block. Sections (3 μm thick) were prepared as positive (MDA-MB-134) and negative (HBL-100) controls were cut from the cell blocks at the same time as the tissue sections.

Cell Line and Tissue Controls. MDA-MB-134 cultured human breast cancer cells processed into sections suitable for IHC staining were used as a positive control and technical (no primary antibody incubation) control for each staining cohort (22). Similarly treated HBL-100 cells were used as a negative control (22). Cell blocks of other cell lines were used to standardize the scoring of IHC staining. Sections of a cancer established as positive in an early staining cohort were included in several staining runs as a positive tissue control. Fresh sections could not be cut for a positive tissue control for each staining run because this would have led to rapid loss of the block. Five sections were cut at a time from this block and stored at 4°C until required.

Quantitation Assesment/Staining Score. To determine the most accurate method of quantitating protein expression by IHC, we measured cyclin D1 protein expression by Western blot analysis of whole-cell lysates of exponentially growing cultured human breast cancer and normal epithelial cells with a range of cyclin D1 expression (HBL 100, MDA-MB-231, T-47D, MCF-7, MDA-MB-134, and HMEC-184; Ref. 22) and compared these results to IHC assessment using various quantitation techniques. Cell blocks of the same cell lines, passaged from the
same original flask and grown concurrently under identical conditions, were used for IHC assessment. IHC scoring was done using the percentage of positive nuclei or the staining intensity of positive nuclei using image analysis of optical or color density.

**Protein Lysates and Western Blots.** Monolayers of cells from replicates of the flasks used to prepare the cell blocks were washed in ice-cold PBS then scraped into ice-cold lysis buffer [50 mM HEPES (pH 7.4), 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 200 μM sodium orthovanadate, 10 mM sodium fluoride, and 100 mM NaF]. The cells were incubated on ice and vortexed, the lysate cleared by centrifugation (14,000 × g for 5 min), and the supernatant was stored at −70°C until use. For Western blots, equal concentrations of whole-cell protein lysates were separated electrophoretically on a 10% polyacrylamide gel and transferred to a nitrocellulose filter. Protein loading was checked with Ponceau S staining. Nonspecific binding was blocked with 5% BSA in Tris-buffered saline (TBS)/Triton (10 mM Tris (pH 7.4), 150 mM NaCl, and 0.1% Triton X-100), followed by incubation for 2 h at room temperature in primary antibody [DCS-6 (23, 25) 1:100 TBS/BSA]. Filters were then incubated with appropriate secondary antibody 1:2000 in 10% skim milk before enhanced chemiluminescence detection (NEN Dupont, Boston, MA). Densitometric analysis of autoradiographs was performed using a Molecular Dynamics (Sunnyvale, CA) Personal Densitometer SI and IP LabGel analysis software (Signal Analytics, VA).

**Cell Counts.** For estimation of the percentage of positive nuclei, cells were considered positive for cyclin D1 expression if their nuclear staining was greater than that seen in the negative control. Counts were performed on low power fields (×25) on a minimum of 200 cells. A QS system for cell counts was used: QS, 1 (≤5%, specimen considered negative); QS, 2 (6–25%); QS, 3 (26–50%); QS, 4 (51–75%); and QS, 5 (76–100%; Ref. 37).

**Staining Intensity and Image Analysis.** Integrated gray scale intensity was measured for captured field images (×200) with the lower threshold set greater than the mean obtained from control sections with no primary antibody incubation (32) using a light microscope (Leica DMRB) with removal of all filters from the light path, a three-chip color video camera (Sony 3CCD-DXC-930), and a Leica Q500MC Image Analysis system according to the manufacturer’s instructions. In color analysis, integrated RGB intensities were obtained on captured field images (×200) with thresholds set to exclude mean RGB intensities detected for a control section with no primary antibody incubation (33).

**Statistical Analysis.** The cyclin D1 QS staining scores were compared using Mann-Whitney U analysis to compare the different histopathological groups with each other. Kruskal-Wallis analysis to assess the relationship between all of the samples, and χ² to compare the percentage of positive ductal and lobular IC lesions. Values for cyclin D1 protein expression obtained by Western blot analysis and IHC counting and intensity were compared by simple linear regression analysis. Analyses were performed using the software program StatView SE + Graphics (Abacus Concept, Inc., Berkley, CA).

**Histopathology.** Serial sections were cut for IHC, and routine H&E staining was performed for all blocks. H&E sections were reviewed by a single histopathologist (A. S. F.), and the diagnosis was confirmed in correlation with the findings of the original histopathologist according to standardized criteria (37). Of a total of 471 sections considered suitable for IHC staining assessment, 60 were diagnosed as normal, 44 PDWA, 33 ADH, 39 low-grade DCIS, 48 high-grade DCIS, and 205 IC, comprising 150 ductal and 55 lobular lesions. For the purposes of this study, DCIS was divided into high-grade and low-grade lesions. “High-grade” lesions were defined as having predominantly high nuclear grade, usually with features of central necrosis and calcification. “Low-grade” lesions encompassed those with low or intermediate nuclear grade, minimal or absent central necrosis, and usually absent or secretory calcification.

**RESULTS**

To develop a quantitative assessment of cyclin D1 gene expression by IHC, we selected six breast cell lines with a known spectrum of expression of cyclin D1 (22) and compared the levels of protein expression by Western blotting and IHC. The data presented in Fig. 1 demonstrate that assessment of protein expression by the QS score, based on the percentage of positive cells in a quartile-based system (QS, 2–5) with those samples with <5% positive cells considered negative (QS, 1) was significantly correlated (r² = 0.91; P = 0.003) with the Western blot data generated from lysates from the same material used to prepare the cell blocks. When staining was assessed either as integrated gray scale or color intensity, the relationship with antigen expression measured by Western blot was poorly correlated with IHC scores.
correlated and did not reach statistical significance (Western blot versus gray scale; $r^2 = 0.549; P = 0.092$; Western blot versus RGB scale, $r^2 = 0.491, P = 0.12$). Consequently, for all subsequent measurements of cyclin D1 by IHC, we used the QS score.

Freshly cut sections from cell blocks of high (MDA-MB-134; QS, 5) and low (HBL-100; QS, 1) cyclin D1-expressing cells were used throughout as positive and negative controls. Positive and negative breast tissue sections were also used as a quality control measure intermittently due to the paucity of this material. Although the positive controls remained positive throughout the study, in some later cohorts, the intensity of staining declined after a period of storage at 4°C, as has been reported recently for other tumor markers, particularly p53 (34). Thus, freshly cut cell block and tissue sections were used as controls throughout.

When the breast tissue sections were assessed, a positive staining pattern was detected in 7 of 60 (11.7%) normal tissue samples derived from reduction mammoplasty procedures. Proliferative lesions were positive in 11 of 44 (25%) cases of PDWA and in 13 of 33 (39.4%) cases of ADH. Low-grade DCIS was positive in 17 of 39 (43.6%) cases, whereas 23 of 48 (47.9%) cases of high-grade DCIS lesions stained positively. In agreement with studies published previously from a number of different laboratories, 48.3%, i.e., 99 of 205 invasive breast cancers, expressed detectable levels of cyclin D1, including 61.8% (34 of 55) lobular and 43.3% (65 of 150) ductal lesions (22-28). Typical examples of the pattern of staining within each of these different histological types is illustrated in Fig. 2, where positive staining is confined to the nuclei of epithelial cells.

Statistical analysis of these data using the Mann-Whitney U test (Fig. 3; Table 1) revealed that cyclin D1 expression in PD was significantly greater than that in NBE ($P = 0.006$). A significant increase in expression was also observed with progression from PD to DCIS ($P = 0.038$). Expression did not significantly increase thereafter with the progression from DCIS to IC ($P = 0.52$). Similarly, there was no significant change in expression from PDWA to ADH ($P = 0.17$) or low-grade to high-grade DCIS ($P = 0.78$). The percentage of lobular carcinomas overexpressing cyclin D1 was significantly greater than ductal lesions ($P = 0.019, \chi^2$ analysis). The increase in expression over the range of histological diagnoses in the whole sample population was also statistically significant ($P = 0.008$) as determined by Kruskal-Wallis analysis, i.e., the level of cyclin D1 expression was significantly related to the histological diagnosis in this population. The QS score or degree of overexpression, as determined by percentage of positive cells in the positive samples, also increased with more malignant phenotypes ($P = 0.001$, Kruskal-Wallis; Table 1).

**DISCUSSION**

IHC has enabled us to examine cyclin D1 protein expression in a relatively large number of samples while preserving the cytological and tissue architecture necessary to confirm the diagnosis in early epithelial lesions. Comparison of quantitation of protein expression by Western blot analysis, IHC count, and IHC nuclear staining intensity has allowed us to adopt a scoring system for IHC, based on percentage of positive nuclei, which is reproducible and correlates significantly with measurement of protein overexpression in whole-cell lysates determined by densitometric analysis of Western blot. By contrast, we found no significant correlation between the Western blot results and staining intensity, either by gray scale or color analysis. This may be a reflection of biological factors peculiar to cyclin D1, such as the cell cycle variation seen in cyclin D1 protein expression (25) or a function of features of IHC, such as antibody binding properties and washing stringency. Alternatively, aspects of the optical system, such as the linearity of the gray scale obtained using a three-chip color camera, can influence measurements of intensity. Nuclear pleomorphism may also influence the intensity of nuclear staining in different cellular populations.

Fig. 3 Mean quartile scores (QS) with SE bars for cyclin D1 expression detected by IHC in breast tissues of different pathologies. Cyclin D1 protein in breast tissue was detected by IHC and scored on the basis of percentage of positive nuclei. Significant increase in cyclin D1 expression was seen between normal breast and PD ($P = 0.006$, Mann-Whitney U) and PD and DCIS ($P = 0.038$) but not between PDWA and ADH ($P = 0.17$), low- and high-grade DCIS ($P = 0.78$), or DCIS and IC ($P = 0.52$). There was a significant upward trend over the whole sample ($P = 0.0001$, Kruskal-Wallis).

This work demonstrates a significant increase in cyclin D1 protein expression with the overall histological advance from NBE to breast cancer. This increase is seen both in the number of positive specimens and the degree of overexpression, as determined by the percentage of positive cells, reflected in the QS of the positive specimens. Individually significant increases in expression were also demonstrated from NBE to PD and PD to DCIS but not between different types of PD or DCIS or at the
transitions from in situ to invasive cancer. Interestingly, a significantly greater number of lobular than ductal IC lesions overexpressed cyclin D1, suggesting that cyclin D1 may play differing roles in distinctly different histological types of breast cancer. Overall, these findings support the view that cyclin D1 overexpression is important throughout breast oncogenesis and raise fundamental questions concerning the functional contribution of cyclin D1 to the development of malignancy. Although examination of the mRNA expression by ISH has suggested previously that cyclin D1 protein expression is actually dysregulated in the earliest proliferative lesions in the breast. This finding is more in keeping with the present understanding of the physiology of cyclin D1 expression, the cyclin D1 transgenic mouse phenotype, and the prevailing paradigm and histological model of breast oncogenesis. Dysregulation of the cell cycle and abnormal cellular proliferation are thought to be integral to the early stages of cancer development (38) and cyclins, and their catalytic partners are recognized as playing a key role in cell cycle regulation (39, 40). An excessive positive drive to cell proliferation from cyclin D1 overexpression may be one explanation for the apparent early oncogenic effects of this gene.

Although amplification of the CCND1 gene encoding cyclin D1 on chromosome 11q13 is only seen in some 13% of primary breast cancers (41), protein overexpression has been demonstrated in 48.3% of tumors in this study, suggesting an amplification-independent mechanism of overexpression (42). IHC on tissue samples has shown reproducible results in several large series, with between 37% and 58% of primary breast cancers staining positive (22–27). These results correlate with the 45% mRNA overexpression seen by Northern analysis of primary breast cancers (22). IHC analysis of DCIS detected cyclin D1 overexpression in 47.9% of 48 high-grade lesions in this study. Similarly, comparison of cyclin D1 overexpression in DCIS and IC components of the same tumor showed 98% correlation in 96 samples assessed by Bartkova et al. (23). In contrast, ISH assessment of mRNA expression detected overexpression in malignant breast epithelium at levels >80% but in only 18.2% of 22 cases of PD (29), whereas we have detected protein overexpression in 31.2% of 77 PD lesions. Although this apparent difference in mRNA and protein expression may, in part, be explained by differences in the sensitivity and specificity of the techniques, the question arises of loss of some form of posttranscriptional control of cyclin D1 protein expression at the transition from NBE to PD with loss of transcriptional control of cyclin D1 mRNA expression from proliferative epithelium to malignancy.

Physiological control of cyclin D1 expression is not presently well understood. The G1 cyclins are thought to mediate the interaction between extracellular mitogens and the drive to cell division (40). Cyclin D1 is induced as part of the delayed early response to cell mitogens, such as insulin and progestin, in T47D breast cancer cells growth arrested by serum deprivation (14). Cyclin D1 mRNA is rapidly down-regulated in MCF-7 ER-positive breast cancer cells after administration of antiestrogens (43) and up-regulated after administration of estrogen in growth-arrested MCF-7 cells (21). Estrogen treatment of growth-arrested MCF-7 cells leads to a greater fold protein than mRNA induction of cyclin D1, suggesting the possibility of both transcriptional and posttranscriptional effects of estrogen stimulation (21). D-type cyclin overabundance due to enhanced protein stability has been demonstrated in human sarcoma cells (44), and posttranscriptional regulation of cyclin D1 by translation initiation factor 4E has been demonstrated in human fibroblasts in culture (45). Cyclin D1 expression is significantly correlated with ER positivity in breast cancers (19, 20), and it is possible that posttranscriptional dysregulation of cyclin D1 expression may form a component of the pathway of oncogenesis associated with estrogen stimulation of the breast.

In conclusion, the present study establishes that cyclin D1 protein expression is present from the earliest stages of breast cancer development, significantly above that seen in NBE, and increases significantly with the progression to invasive cancer. We have not addressed the relationship between cyclin D1 expression in proliferative or DCIS lesions and the risk of progression to invasion. Nor have we investigated the implications of cyclin D1 expression in the prognosis of invasive breast cancer, although these questions are being addressed in ongoing research. The results of this study strongly support the theory that loss of control of cyclin D1 expression is a critical early event in breast oncogenesis, and we have suggested mechanisms that may be responsible for this molecular abnormality and their potential functional consequences. If these hypotheses can be sustained, measurement of cyclin D1 expression may be a clinically important measure of disease phenotype, therapeutic responsiveness, and patient outcome in proliferative and noninvasive malignant breast lesions. Furthermore, if overexpression of cyclin D1 plays a causative role, it may provide a new target for therapeutic intervention in the earliest stages of breast on-
cogenesis. Further investigation of the molecular mechanisms underlying dysregulation of cyclin D1 gene expression and its functional consequences in the human breast appear crucial to understanding the evolution of breast cancer.

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Cyclin D1 protein is overexpressed in hyperplasia and intraductal carcinoma of the breast.

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