Cyclin E, a Potential Prognostic Marker for Breast Cancer

Khandan Keyomarsi, Nuala O'Leary, Gyongyi Molnar, Emma Lees, Howard J. Fingert, and Arthur B. Pardee

Division of Cell Growth and Regulation, Dana-Farber Cancer Institute, and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115 [K. K., N. O., G. M., A. B. P.]; Massachusetts General Hospital Cancer Center, Charlestown, Massachusetts 02129 [E. L.]; and St. Elizabeth's Medical Center, Boston, Massachusetts 02135 [H. J. F.]

Abstract

A fundamental cause of cancer is changed properties of genetic material, which may deregulate normal development of the tissue or provide selective growth advantage to the tumor cell. This deregulation of cell proliferation results from altered production of a handful of proteins that play key roles in progression through the eukaryotic cell cycle. Some of these proteins include tumor suppressor genes or oncogenes.

However, no one general change or alteration of a critical gene has yet been found in all cancers. Using surgical material obtained from patients with various malignancies, we show that breast cancers and other solid tumors, as well as malignant lymphocytes from patients with lymphatic leukemia, show severe quantitative and qualitative alterations in cyclin E protein production independent of the S-phase fraction of the samples. Hence, these alterations represent a true difference between normal versus tumor tissue. In addition, in breast cancer, the alterations in cyclin E expression become progressively worse with increasing stage and grade of the tumor, suggesting its potential use as a new prognostic marker.

Introduction

Breast cancer is a major killer worldwide. One in eight American women may expect some form of breast cancer in their lifetime (1). Success in treatment depends greatly upon early detection. In breast cancer, the tumor size, grade, stage, lymphatic or vascular invasion, axillary node metastases, and hormone receptors are well established but not definitive prognostic factors (2). Thus, additional prognostic factors are essential.

Attention has recently focused on molecular markers, in particular genes involved in the pathogenesis of breast carcinoma. The first such marker to be tested for its clinical relevance is the c-erb B2/neu oncogene, whose activation can occur by amplification of c-erb B2 DNA and by overproduction of its mRNA and protein (3). Approximately 20% of breast carcinomas show evidence of c-erb B2 activation, which correlates with a poor prognosis primarily in patients with metastasis to axillary lymph nodes. Attempts to correlate c-erb B2 activation with other prognostic factors in breast carcinoma have, however, reported conflicting conclusions (4). Since it is likely that breast cancer is a result of a progressive accumulation of many different somatic mutations in diverse genes such as oncogenes and tumor suppressor genes, the clinical relevance of new markers must be examined carefully.

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2 To whom requests for reprints should be addressed, at New York State Department of Health, Wadsworth Center for Laboratories and Research, Empire State Plaza, Room C-362, P. O. Box 509, Albany, NY 12201-0509.

With the discovery of cyclins and cyclin-dependent kinases, it is now possible to specifically ask whether the deregulation of any or all of these molecules could lead to oncogenesis. The link between oncogenesis and cyclins was made with discovery of the inappropriate expression of two cyclins in tumors (5, 6): (a) cyclin A gene is the site of integration of a fragment of the hepatitis B virus genome in a hepatocellular carcinoma (7). Cyclin A is also associated with the adenovirus transforming protein E1A in adenovirus transformed cells (8–10); (b) in some parathyroid tumors, the Pradl (cyclin D1) locus is overexpressed as a result of a chromosomal rearrangement that translocates it to the enhancer of the parathyroid hormone gene (11–13). Pradl has also been found to reside near the B-lymphoid tumor-associated chromosome 11 breakpoint known as BCL1, activated by t(11;14) translocations and targeted by chromosomal translocations in centrocytic lymphomas (14). The same locus undergoes gene amplification in mouse skin carcinogenesis, as well as breast, esophageal, colorectal, and squamous cell carcinomas (15–20). These observations describe occasional changes involving only two cyclins. They do not provide a clear connection between general derangements of other cyclins or their dependent kinases in a single tumor type.

Recently, we have correlated the deranged expression of cyclins in general to loss of growth control (21). Using proliferating normal versus human tumor breast cell lines in culture as a model system, we have described several changes that are seen in all or most of these lines. These alterations include (a) increased cyclin mRNA stability, resulting in (b) general overexpression of mitotic cyclins and of CDC2 RNAs and proteins in 9 of 10 tumor lines, and (c) deranged order of appearance of cyclins in synchronized tumor versus normal cells, with mitotic cyclins appearing prior to G1 cyclins. The most striking abnormality in cyclin expression was that of cyclin E. We found an 8-fold amplification of cyclin E gene in one tumor line, 64-fold overexpression of its mRNA, and altered expression of its protein. In addition, cyclin E protein was not only overexpressed in all (10 of 10) breast tumor cell lines examined but was present in different sizes than found in normal cells.

In this report, we extended these observations to the in vivo condition by examining the pattern of cyclin E protein expression in tumor and normal adjacent tissue obtained from breast cancer patients. We find that the altered expression of cyclin E protein occurred in most of the breast tumor tissues examined, and its alterations increased with increasing grade and stage of the tumor. Cyclin E alteration was also more consistent than c-erb B2 overexpression in breast cancer. Furthermore, cyclin E was also altered in other types of solid tumors as well as leukemia. This alteration is a true normal versus tumor difference, independent of the S-phase fraction of the tumor as measured by the expression of PCNA. These observations suggest that cyclin E may potentially be used as a prognostic marker in breast cancer, in addition to other markers such as c-erb B2 currently used in the clinic.

3 The abbreviations used are: PCNA, proliferating cell nuclear antigen; CLL/PL, chronic lymphocytic leukemia with 50% prolymphocytes; NAT, normal adjacent tissue.
Materials and Methods

Western Blot Analysis of Tissue Samples. Snap frozen surgical specimens from patients diagnosed with breast cancer were obtained from the National Disease Research Interchange/Cooperative Human Tissue Network, Eastern Division. Detailed pathology/surgical reports summarize the source of specimens, clinical history and diagnosis, gross description, summary of sections, final diagnosis, and if available, estrogen/progesterone receptor status, DNA ploidy, and oncogene data. Approximately 0.5-5 g of each matched tissue (NAT and tumor tissue obtained from the same patient) were added to 1 volume of sonication buffer containing a cocktail of proteases and phosphatase inhibitors (22) in a low salt buffer, homogenized for 1 min with a hand-held homogenizer, and sonicated at 4°C using a microtip adapter for 1 min. Homogenates were centrifuged at 100,000 × g for 45 min at 4°C. The supernatants were assayed for protein content (23), aliquoted, stored at −70°C, and subjected to Western blot analysis as described (21, 24). Briefly, 100 µg of protein from each tissue sample was electrophoresed in each lane of a 10% sodium dodecyl sulfate-polyacrylamide gel (cyclin E, cyclin D1, and PCNA) or 5% sodium dodecyl sulfate-polyacrylamide gel (c-erb B-2) and transferred to Immobilon-P. Blots were incubated with blocking buffer (20 µg Tris-HCl, pH 7.5-150 µg NaCl-5% dried milk-0.2% Tween) overnight at 4°C and then incubated with various primary antibodies diluted in blocking buffer. Rabbit anti human cyclin E serum at a dilution of 1:2500 (25), monoclonal antibody HE12 to cyclin E at a dilution of 1:10 (26), polyclonal antibody to c-erb B2 (Oncogene Science) at a dilution of 1:30, polyclonal antibody to cyclin D1 at a dilution of 1:250 (12), and monoclonal antibody PC10 to PCNA (Santa Cruz Biochemicals) at a dilution of 1:100 were incubated for 3 h. The blots were then washed and incubated with either goat anti mouse or anti-rabbit horse radish peroxidase conjugate at a dilution of 1:5000 in blocking buffer for 1 h, washed extensively, and developed with detection reagents (ECL) supplied by Amersham Biochemicals. ECL exposures for all Western blots were of similar duration, i.e., 1–10 s.

Blood Samples. Whole blood samples were obtained from a normal volunteer and CLL/PL patients. Monocytes/lymphocytes were purified by Ficoll-Paque gradient centrifugation as described (27), homogenized by sonication, and subjected to Western blot analysis. Staining of peripheral blood lymphocyte preparation was performed by the Wright-Giemsa technique.

Results and Discussion

Abnormal Expression of Cyclin E Protein in Human Breast Tumor Tissue. An important experimental question concerns the applicability of culture studies with cell lines to the in vivo condition. We directly examined the relevance of cyclin derangement to in vivo conditions by measuring the expression of cyclin E protein as compared to c-erb B2 in breast tumor samples versus normal adjacent breast tissue obtained from patients diagnosed with breast cancer (Fig. 1). Whole cell lysates were initially prepared from nine paired cases of human breast carcinomas and adjacent nontumorous tissues and subjected to Western blot analysis using two different antibodies to cyclin E, one to c-erb B2 (Fig. 1A), one to cyclin D1, and one to the PCNA (Fig. 1B). These analyses revealed several findings.

(a) Cyclin E protein is abnormally expressed quantitatively and qualitatively in most tumor tissues compared to the NAT. In the tumor samples, cyclin E antibody reacted strongly with at least three over-expressed proteins ranging in size from Mr 35,000-50,000, whereas in the NAT samples, one major protein of ~Mr 50,000 was present at very low levels. Both the monoclonal and polyclonal antibodies specific to cyclin E recognized the same multiple cyclin E bands, even though the two anti-E antibodies recognize different epitopes on the protein (25, 26, 28). These in vivo observations are consistent with results obtained with cultured breast cell lines (21). Thus, abnormal

Fig. 1. Abnormal expression of cyclin E protein and c-erb B2 in human breast tumor vs. normal adjacent tissue. Whole cell lysates were extracted from nine pairs of normal adjacent and breast cancer tissues. (Each paired sample was obtained from the same patient). Breast cancer types and histological/tumor grades are: Lane 1, metastatic ductal carcinoma, grade IV; Lane 2, invasive, poorly differentiated ductal carcinoma of the breast, grade III; Lane 3, infiltrating ductal carcinoma, grade N/A; Lane 4, Poorly differentiated metastatic ductal carcinoma, grade IV; Lane 5, inflammatory breast cancer, grade II/III; Lane 6, malignant phylloides tumor, Grade II/III; Lane 7, infiltrating and in situ ductal carcinoma, Grade II; Lane 8, small foci of in situ ductal carcinoma, grade I/II; Lane 9, infiltrating ductal carcinoma, grade III/IV. Protein extracts were analyzed on Western blots after boiling extraction (100 µg of protein extract/lane) and hybridized with the indicated antibodies. (A) Large arrowhead, cyclin E protein of the predicted size of ~Mr 50,000. Small arrowheads, the two extra cyclin E-like proteins observed in the tumor tissue samples at Mr 42,000 and 35,000. (B) Arrows, a Mr 35,000 cyclin D1 protein in the left panel and a Mr 36,000 PCNA protein in the right panel. Molecular mass standards were used on each gel to estimate the position of each band.
expression of cyclin E is a general phenomenon associated with oncogenesis of breast cancer. 

(b) Cyclin E is abnormally expressed in 8 of 9 tumor tissue samples (with the exception of Fig. 1A, Lane 8), but c-erb B2 is overexpressed in only 3 of 9 of these cases (Fig. 1A, Lanes 4, 6, and 9), suggesting that cyclin E is a more sensitive and consistent marker for prognosis of breast cancer than the commonly used c-erb B2. We found a similar overexpression of both c-erb B2 and cyclin E in samples 4 and 9, both from patients with advanced metastatic breast cancer.

(c) Analysis of cyclin D1 revealed a different pattern of overexpression in these breast tumor samples (Fig. 1B) as compared to cyclin E (Fig. 1A). The cyclin D1 locus has been shown to be amplified in 15–20% of breast cancer samples; however, no attempt has been made to correlate the overexpression of cyclin D1 protein to progression of the disease (16, 17, 29, 30). In our analysis, cyclin D1 overexpression in 2 of 9 cases (Fig. 1B, Lanes 2 and 3) occurs in a different subtype of breast cancers which do not correlate with overexpression of c-erb B2 or cyclin E (Fig. 1A, Lanes 4 and 9).

(d) Cyclins are expressed in a cell cycle regulatory manner and hence their expression is higher in proliferating than in nonproliferating quiescent cells. To determine whether the altered expression of cyclin E observed in these tissues is independent of cell proliferation, we also measured the expression of PCNA. PCNA, a Mr 36,000, nonhistone nuclear protein, is essential for cellular DNA synthesis and is closely linked to the cell cycle (31–33). The breast NAT samples have a very low cell proliferative activity as apparent by the faint PCNA signal observed on Western blots using a monoclonal antibody to PCNA (PC10; Fig. 1B). The expression of PCNA was much higher in the tumor samples, indicative of their high S-phase fraction. However, there was very little difference, if any, in PCNA expression among these different tumor tissue samples, regardless of the type of cancer, the stage of disease, or the degree of altered cyclin E expression. The altered expression of cyclin E seems to be distinct from the proliferative index of the tumor, and is a true normal versus tumor difference (see below).

**Altered Expression of Cyclin E Correlates with Increased Breast Tumor Stage and Grade.** To examine the likelihood that the altered expression of cyclin E is associated with high tumor stage and grade and therefore poor prognosis, we analyzed an additional seven paired samples of human breast carcinomas and NAT which are present in the tumor samples, most pronounced in lung, stomach, and kidney samples. Hence, as with the breast cancer specimens, cyclin E differences observed between NAT and other tumor samples is more consistent than the proliferative activity of the tissue and could represent

stage T1/N0/M0, grade I patients; Fig. 2, Lane 3 from a tumor stage T2/N0/M0, grade I patient; Fig. 2, Lane 4 from a tumor stage T3/N0/M0, grade II patient; Fig. 2, Lane 5 from a tumor stage T2/N1/M0, grade II/III patient; Fig. 2, Lane 6 from a tumor stage T3/N1/M0, grade II/III patient; and Fig. 2, Lane 7 from a tumor stage T3/N0/M0, grade III patient. In all the NAT samples, only cyclin E protein of ~Mr 50,000 was present at very low levels. In low stage and grade tumors, cyclin E antibody recognized three proteins of low abundance ranging in size from Mr 35,000–50,000 (Fig. 2, Lanes 1–3). These proteins become progressively more abundant and altered with increasing tumor grade and stage (Fig. 2, Lanes 4–7).

To assess whether the increase in expression of cyclin E is correlated with increased proliferation rates, PCNA protein levels were also measured and revealed that there is only a minor (2–3 fold) increase in PCNA levels from low to high stage and grade tumors, not enough to account for the massive differences in cyclin E expression. Furthermore, even though cyclin E protein was altered and overexpressed in all four of the higher stage grade tumors, c-erb B2 was overexpressed in only two of the samples. Thus, quantitatively and qualitatively, increased alterations of cyclin E protein correlates with increased tumor stage and grade more consistently than the proliferative index of the cells or overexpression of c-erb B2.

**Alteration of Cyclin E Is Associated with Other Solid Tumors.** Cyclin E is a cell cycle-specific protein whose normal function is thought to regulate the late G1 to S transition in all mammalian cells, suggesting that its alterations should not be unique to breast cancer. We therefore examined whole cell lysates prepared from patients diagnosed with metastatic lung, kidney, pancreas, stomach, colon, uterus, testis, endometrial, esophagus, melanoma (spleen), and ovarian cancers (Fig. 3, A and B). In half of the cases, NAT samples were also available for analysis. Western blots were analyzed using cyclin E and PCNA antibodies. In every case examined, there were severe alterations of cyclin E expression in the tumor samples compared to the NAT samples. Although the NAT samples did not contain any detectable tumor tissue, as indicated in the pathology reports, the normal tissues contained proliferating cells, probably due to local inflammation, as evident by high PCNA levels (Fig. 3A). Moreover, the differences in PCNA levels in NAT versus tumor samples were not sufficient to account for the observed severe cyclin E alterations seen in the tumor samples, most pronounced in lung, stomach, and kidney samples. Hence, as with the breast cancer specimens, cyclin E differences observed between NAT and other tumor samples is more consistent than the proliferative activity of the tissue and could represent
a true normal versus tumor difference. Collectively, these observations suggest that cyclin E alterations are of general occurrence, irrespective of the tumor origin.

Cyclin E alterations and PCNA levels were also examined in whole cell lysates prepared from surgical samples obtained from several prostate cancer patients and subjected to Western blot analysis and are shown according to Gleason's grade (a histological score used in the pathological description of prostate cancer due to its correlation with subsequent metastases and prognosis; Fig. 3C). These analyses revealed that, in the prostate NAT samples, cyclin E was present at very low to undetectable levels. However, there were at least two isoforms of cyclin E protein present in most tumor samples examined, and the expression level of these isoforms increased with increasing tumor grade (with the exception of Fig. 3, Lane C5). There was a high cellular proliferative activity in the hyperplastic NAT samples as evident by the PCNA levels. The PCNA levels increased with increasing tumor grade; however, the difference in cyclin E expression between NAT and tumor tissue was higher than that of PCNA. These observations suggest that cyclin E alteration is a general event which occurs in tumor and not proliferating normal cells.

Alteration of Cyclin E in Lymphatic Leukemia. In order to assess the generality of cyclin E alteration in cancer and in non-solid tumors, we next examined blood samples from leukemia patients for the presence of cyclin E alterations. Fig. 4A depicts Wright's stain of peripheral blood lymphocytes from a normal (N) volunteer and the three CLL/PL patients (L1-3). Whole cell lysates prepared from lymphocytes, isolated by centrifugation of whole blood through ficoll gradient, were subjected to Western blot analysis (Fig. 4B). In the normal volunteer, the pattern of expression of cyclin E protein is indicative of nonmalignant tissue as evident by very low levels of different cyclin E isoforms. In the CLL/PL patient with stage A form of the disease, there was a shift in the form of cyclin E protein expression but very little, if any, alterations. Only the Mr 50,000 cyclin E protein was moderately overexpressed. In the patient with stage B of the disease, however, there was a strong overexpression of the Mr 50,000 cyclin E protein and the appearance of the lower molecular weight isoforms of the protein. Finally, cyclin E alteration was most pronounced in a stage C CLL/PL patient, as evident by the overexpression of proteins ranging in size from Mr 35,000-50,000 (Fig. 4B, Lane L3 and L3 (TX)). There was no difference in the pattern of cyclin E protein alteration before (L3) or 6 days into a 7-day treatment with infusion of 2-chlorodeoxy-adenosine [L3 (TX)], suggesting that the cyclin E producing leukemia cells were not preferentially killed during this treatment, even though the total number of lymphocytes was considerably reduced. PCNA levels in all the samples were quite similar, indicating that there are proliferating cells at every stage of the disease as well as in the normal lymphocytes. Thus, PCNA measured only cellular proliferative activity and did not differentiate between normal versus tumor cells.

In summary, we have examined the alterations of cyclin E protein in surgical material from 42 cancer patients and (a) have corroborated our previous in vitro observations (21) using cultured normal versus tumor breast cells by extending them to the in vivo condition. We observe that cyclin E undergoes very similar alterations in vivo as in vitro; therefore, the overexpression and alteration of cyclin E in is not an artifact of culture conditions and represents an in vivo phenomenon; (b) our data suggest that the alteration of cyclin E becomes more severe with breast tumor stage and grade and is more consistent than cell proliferation or other tumor markers such as c-erb B2 or cyclin D1. Hence, the altered expression of cyclin E in the breast tumor...
samples is not a mere consequence of cell proliferation and represents a true difference among NAT and low and high stage and grade tumors and as such represents a potential new prognostic marker for breast cancer; and (c) alteration of cyclin E is a general phenomenon which occurs in all 12 kinds of solid tumors examined as well as blood samples from patients with chronic lymphocytic leukemia exhibiting a prolymphocytic morphology.

Future studies should delineate the mechanism of this alteration in the tumor cells and examine the consequences of cyclin E alterations in the transformation process. The general alterations of cyclin E protein observed in most tumor samples examined is suggestive of its role as an oncogene. Recent in vitro studies propose that cyclin E alterations in tumor cells may be in part due to gene amplification and deletional mutations of the cyclin E gene. Furthermore, in synchronized populations of tumor cells which amplify the cyclin E gene, cyclin E protein is no longer cell cycle regulated and appears constitutively in the cell cycle. Finally, to examine the potential use of cyclin E clinically as a prognostic marker in cancer, prospective studies with a larger number of patients are required.

K. Keyomarsi et al., unpublished observations.

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