

## CYCLIN E AND SURVIVAL IN PATIENTS WITH BREAST CANCER

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### ABSTRACT

**Background** Cyclin E, a regulator of the cell cycle, affects the behavior of breast-cancer cells. We investigated whether levels of cyclin E in the tumor correlated with survival among patients with breast cancer.

**Methods** Tumor tissue from 395 patients with breast cancer was assayed for cyclin E, cyclin D1, cyclin D3, and the HER-2/*neu* oncogene with the use of Western blot analysis. Full-length, low-molecular-weight, and total cyclin E were measured. Immunohistochemical assessments of cyclin E were also made of 256 tumors. We sought correlations between levels of these molecular markers and disease-specific and overall survival.

**Results** The median follow-up was 6.4 years. A high level of the low-molecular-weight isoforms of cyclin E, as detected by Western blotting, correlated strongly with disease-specific survival whether axillary lymph nodes were negative or positive for metastases ( $P < 0.001$ ). Among 114 patients with stage I breast cancer, none of the 102 patients with low levels of cyclin E in the tumor had died of breast cancer by five years after diagnosis, whereas all 12 patients with a high level of low-molecular-weight cyclin E had died of breast cancer within that period. In multivariate analysis, a high total cyclin E level or high levels of the low-molecular-weight forms of cyclin E were significantly correlated with poor outcome. The hazard ratio for death from breast cancer for patients with high total cyclin E levels as compared with those with low total cyclin E levels was 13.3 — about eight times as high as the hazard ratios associated with other independent clinical and pathological risk factors.

**Conclusions** Levels of total cyclin E and low-molecular-weight cyclin E in tumor tissue, as measured by Western blot assay, correlate strongly with survival in patients with breast cancer. (N Engl J Med 2002;347:1566-75.)

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**T**HE prognosis in patients with newly diagnosed breast cancer is determined primarily by the presence or absence of metastases in draining axillary lymph nodes.<sup>1</sup> However, in approximately one third of women with breast cancer who have negative lymph nodes, the disease recurs, and about one third of patients with positive lymph nodes are free of recurrence 10 years after local-regional therapy.<sup>2,3</sup> These data highlight the need for more sensitive and specific prognostic indicators.

A number of biologic factors have been used to refine risk categories in breast cancer. We have focused on the role of cyclin E in determining the virulence and metastatic potential of tumor cells.<sup>4-8</sup> In normal dividing cells, cyclin E regulates the transition from the G<sub>1</sub> phase to the S phase<sup>9,10</sup>; a high level of the cyclin E protein accelerates the transition through the G<sub>1</sub> phase.<sup>11,12</sup>

The cyclin E gene is amplified and the cyclin E protein is often constitutively expressed in breast-cancer cell lines.<sup>6,8,13,14</sup> Some of these lines overexpress not only the full-length 50-kD cyclin E protein, but also up to five low-molecular-weight isoforms of cyclin E (ranging in size from 34 to 49 kD).<sup>6,7,15</sup> These isoforms, which lack the amino terminus, are hyperactive, as compared with the full-length protein, in phosphorylating substrates and inducing progression from the G<sub>1</sub> phase to the S phase.<sup>16</sup>

Previous studies of the prognostic importance of cyclin E in breast cancer have yielded contradictory results.<sup>17,18</sup> In a retrospective analysis of 278 patients with breast cancer, Porter et al. found that overexpression of the full-length cyclin E protein and low levels of the cyclin-dependent kinase inhibitor p27 correlated with poor survival among patients with node-negative disease.<sup>18</sup> By contrast, in a study of 157 patients with breast cancer, Donnellan et al. reported that the correlation between high levels of cyclin E in the tumor cells and poor outcome lost its significance in a

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multivariate analysis.<sup>17</sup> The antibody to cyclin E used for immunohistochemical analysis in these studies cannot reliably detect the low-molecular-weight forms of cyclin E. Western blotting with an anti-cyclin E antibody targeted against the C-terminal domain of the protein, however, permits detection of both the full-length and low-molecular-weight isoforms of cyclin E.<sup>6-8,16</sup>

To determine whether levels of these isoforms are associated with outcomes in patients with breast cancer,<sup>7,19-21</sup> cyclin E and its low-molecular-weight isoforms were measured by Western blotting in samples of breast-cancer tissue from 395 patients and by immunohistochemical techniques in samples from a subgroup of 256 patients. The levels were compared with established factors that predict disease-specific and overall survival. Total cyclin E levels and the level of low-molecular-weight forms of cyclin E as measured by Western blotting but not by immunohistochemical analysis proved to be strongly associated with survival among patients with breast cancer.

## METHODS

### Tissue Samples and Study Patients

Tumor tissue was obtained from a centralized reference laboratory (Quantitative Diagnostic Laboratories). A total of 430 samples consisting of a minimum of 100 mg of breast-cancer tissue were available. Each patient had received a diagnosis of breast cancer between 1990 and 1995 at 1 of 12 hospitals in the Chicago area. Specimens were shipped to the Wadsworth Center research laboratories for Western blot analysis. This study was approved by the institutional review board of the Wadsworth Center.

The reference laboratory also provided base-line pathological and demographic data (obtained from the individual hospitals), as well as the steroid-receptor status, the DNA index, and the proliferation index (as described below). Information concerning clinical staging and survival was obtained from the tumor registries of each hospital. Patients whose death was clearly documented to be due to breast cancer were considered to have died of breast cancer; other deaths were considered not to have been caused by breast cancer. The data presented here are from 395 patients for whom data on outcome were available.

### Hormone-Receptor, DNA, and Proliferation Assays

The procedures for the hormone-receptor and proliferation assays have been described elsewhere.<sup>22-24</sup> Specimens were scored as positive for estrogen receptors or progesterone receptors if they contained at least 11 fmol of specific binding sites per milligram of protein.<sup>22</sup> Specimens were considered to have a moderate-to-high proliferative index if 7 percent or more of the nuclei were labeled with anti-Ki-67 antibody.<sup>24</sup> DNA ploidy values were determined by means of image cytometric measurements of nuclear DNA content.<sup>25,26</sup>

### Western Blot Assays

Levels of full-length cyclin E, its low-molecular-weight isoforms, cyclin D1, cyclin D3, and the HER-2/*neu* oncogene were evaluated by Western blot analysis of lysates prepared from specimens of frozen tumor tissue as previously described.<sup>7,27</sup> The primary antibodies used were as follows: monoclonal antibody HE12 to cyclin E,<sup>28</sup> targeting the C-terminal epitope of the protein, diluted 1:10; polyclonal antibody to HER-2/*neu* (Oncogene Science), di-

luted 1:30; monoclonal antibodies to cyclin D1 and proliferating-cell nuclear antigen (Santa Cruz Biotechnology), diluted 1:250; monoclonal antibody to cyclin D3 (Transduction Laboratories), diluted 1:100; and actin monoclonal antibody (Boehringer-Mannheim) at a concentration of 0.63  $\mu$ g per milliliter. Equivalent amounts of protein from two control cell lines (normal mammary epithelial cells and breast-cancer cells) were included on each gel as internal laboratory standards.

The protein levels in the Western blots were measured by densitometric scanning of the corresponding bands with the use of IP-Lab Gel software (Scanalytics). On the basis of the densitometric values, the amounts of cyclin D1, full-length cyclin E, low-molecular-weight cyclin E, and total (full-length plus low-molecular-weight) cyclin E were scored as low (less than or equal to the level of protein found in normal breast epithelium) or high (higher than in the normal-cell controls). Specifically, cyclin E values were given a score of 0 to 3 (for full-length) and 0 to 10 (for low-molecular-weight and total) on the basis of the densitometric values and were then distributed into two clusters — high and low, as compared with values obtained from normal tissues. For full-length cyclin E, expression was scored as high if the value was at least 0.5 (i.e., higher than the highest value for normal breast epithelium); a total of 14 normal tissue samples were examined. For both low-molecular-weight and total cyclin E, specimens with values of 1.2 or higher were classified as high. All normal-cell controls were negative for cyclin D3 and HER-2/*neu*. On Western blotting, densitometric values for these proteins clustered into three groups and were scored as negative, low level, or high level. Densitometric values for actin were used to standardize for equal protein loading among the samples assayed. The Western blot analysis and scoring of the aforementioned biologic markers were performed by investigators who were unaware of the patients' outcomes.

### Immunohistochemical Studies

A subgroup of 256 samples of tumor tissue were available for immunohistochemical analysis with the use of an affinity-purified polyclonal antibody to cyclin E.<sup>5</sup> We used the C-terminal peptide corresponding to amino acids 381 to 411 of cyclin E<sup>16</sup> as an antigen in the affinity purification. This polyclonal antibody is against the same epitope as monoclonal HE12 antibody and can be used in Western blots to detect both the full-length and low-molecular-weight isoforms of cyclin E.<sup>5,7,8</sup> Snap-frozen tissues were embedded in Optimal Cutting Temperature compound, sectioned at 5- $\mu$ m intervals, placed on coated slides, fixed, and stained for cyclin E as previously described.<sup>22-24</sup> At least two representative tissue sections from each patient with breast cancer were examined. Each was scored from 0 to 10 on the basis of the intensity of staining and the percentage of tumor cells stained. In 15 cases, sections of normal tissue were tested along with tumor tissue. Scores for normal breast-cell controls ranged from 0 to 2. The tumor samples were then designated as having either low cyclin E levels (less than or equal to the level of protein found in normal breast epithelium, indicated by a score of less than or equal to 2) or high cyclin E levels (higher than the level in normal-cell controls, indicated by a score of more than 2). Immunohistochemical analysis and scoring were performed at Quantitative Diagnostic Laboratories by an investigator who was unaware of the results of Western blot analysis and of patient outcome.

### Statistical Analysis

Overall survival was calculated from the date of surgical excision of the primary tumor to the date of death or last follow-up. For disease-specific survival, data for patients who died from causes other than breast cancer were censored at the time of death. Overall survival and disease-specific survival curves were computed by the Kaplan-Meier method.<sup>29</sup> Error bars shown on the curves are 95 percent confidence intervals calculated according to the method of Greenwood.<sup>30</sup>

Univariate analyses of disease-specific survival according to levels of total and low-molecular-weight cyclin E and other factors (age, tumor size, nodal status, clinical stage, and various biologic markers) were performed with the use of a two-sided log-rank test.<sup>31</sup> A multivariate analysis of disease-specific survival was performed with the use of the Cox proportional-hazards model<sup>31</sup> with both forward and backward stepwise inclusion of factors, with an inclusion criterion of  $P \leq 0.05$  and an exclusion criterion of  $P > 0.05$ . Each model was fitted twice, once by a predominantly forward procedure in which the initial model contained only a constant term and once by a predominantly backward procedure in which all factors were included in the model at the first step. For both procedures, addition and removal of terms were considered at each step.

## RESULTS

### Characteristics of the Patients

The median age of the study population was 64 years (range, 29 to 95). The majority (92 percent) had stage I, II, or III breast cancer. A total of 67 percent of the total study population and 50 percent of patients with stage I disease had received adjuvant therapy. After a median follow-up of 6.4 years (range, 1.5 to 11.0), 121 of the 395 patients (30.6 percent) had died of breast cancer. The 5- and 10-year disease-specific survival rates for the entire cohort of patients were 71 percent and 62 percent, respectively. Overall survival was 66 percent at 5 years and 47 percent at 10 years. The results of the univariate analysis of disease-specific survival and overall survival according to clinical factors and biologic markers are shown in Table 1. As expected, there was a significant association between clinical stage and outcome.

### Biologic Markers

A representative Western blot analysis for cyclin E, cyclin D1, and proliferating-cell nuclear antigen in tissue samples from 10 patients with breast cancer at various stages is shown in Figure 1A. The three patients with high levels of the low-molecular-weight forms of cyclin E (Patients 2, 6, and 10) died of breast cancer 21, 16, and 14 months after diagnosis, respectively, whereas the other seven patients, with undetectable or low levels of low-molecular-weight forms of cyclin E, were alive at the time of the last follow-up (57 to 75 months after diagnosis), with no evidence of disease. Figure 1B shows expression of cyclin E in 11 patients with early-stage breast cancer. Tissue from Patients 2, 3, 6, 7, and 8 had a low level of cyclin E, and these patients were free of disease at the time of last follow-up (99 to 108 months after diagnosis). Patient 1 died of unrelated causes and had no evidence of disease 23 months after diagnosis. Breast-cancer tissue from the remaining patients had high levels of the low-molecular-weight forms of cyclin E, and these patients died from breast cancer between 33 and 65 months after diagnosis. Some tumors (those of Patient 2 in Fig. 1A and Patient 5 in Fig. 1B) had high levels of the low-molecular-weight forms of cyclin E

but low levels of full-length cyclin E protein. In the entire cohort, there were 10 such patients, 9 of whom died of breast cancer, with a median survival of 1 year (range, 1 month to 6.9 years).

### Univariate Analyses

The five-year overall survival and the five-year disease-specific survival were significantly longer among patients with low levels of low-molecular-weight, full-length, or total cyclin E than among patients whose tumors had high levels of these proteins ( $P < 0.001$  by the log-rank test) (Table 1 and Fig. 2). Figures 2A and 2D show disease-specific survival and overall survival, respectively, according to the level of total cyclin E in the tumor (low or high). The absence of cyclin D1 (Fig. 2B and 2E) and of cyclin D3 (Fig. 2C and 2F) was also associated with improved disease-specific and overall survival, but the correlations were less striking than those for cyclin E.

### Cyclin E and Stage of Disease

The proportion of tumors with high levels of cyclin E increased with increasing stage of disease ( $P < 0.001$  by the chi-square test; data not shown). Figure 3A shows disease-specific survival according to clinical stage and total cyclin E level as determined by Western blotting. In patients with stage I, II, or III breast cancer, a high total cyclin E level was strongly linked to disease-specific survival, but this was not the case among patients with stage IV disease ( $P = 0.35$ ) (Fig. 3D). Of the 114 patients with stage I disease, 12 had a recurrence of breast cancer and died of breast cancer, with a median time to death of 4.1 years (range, 1 to 7). All 12 — and only those 12 of the 114 patients — had a high level of cyclin E in their tumors (Fig. 3A). In contrast, analysis of stage-specific survival according to the cyclin E level measured by immunohistochemical analysis showed a significant association only among patients with stage III disease (data not shown).

### Proportional-Hazards Modeling

We performed proportional-hazards modeling of disease-specific survival and overall survival according to Western blotting and immunohistochemical measures of cyclin E. When they were included individually in separate models, all measures were significantly associated with disease-specific survival and overall survival. High levels of total cyclin E and high levels of low-molecular-weight cyclin E were associated with hazard ratios for death from breast cancer of 33.0 and 20.8, respectively, whereas the hazard ratio for death from breast cancer among patients with high levels of cyclin E as measured by immunohistochemical analysis was 2.90. When all four of these measures of cyclin E were included simultaneously in a proportional-

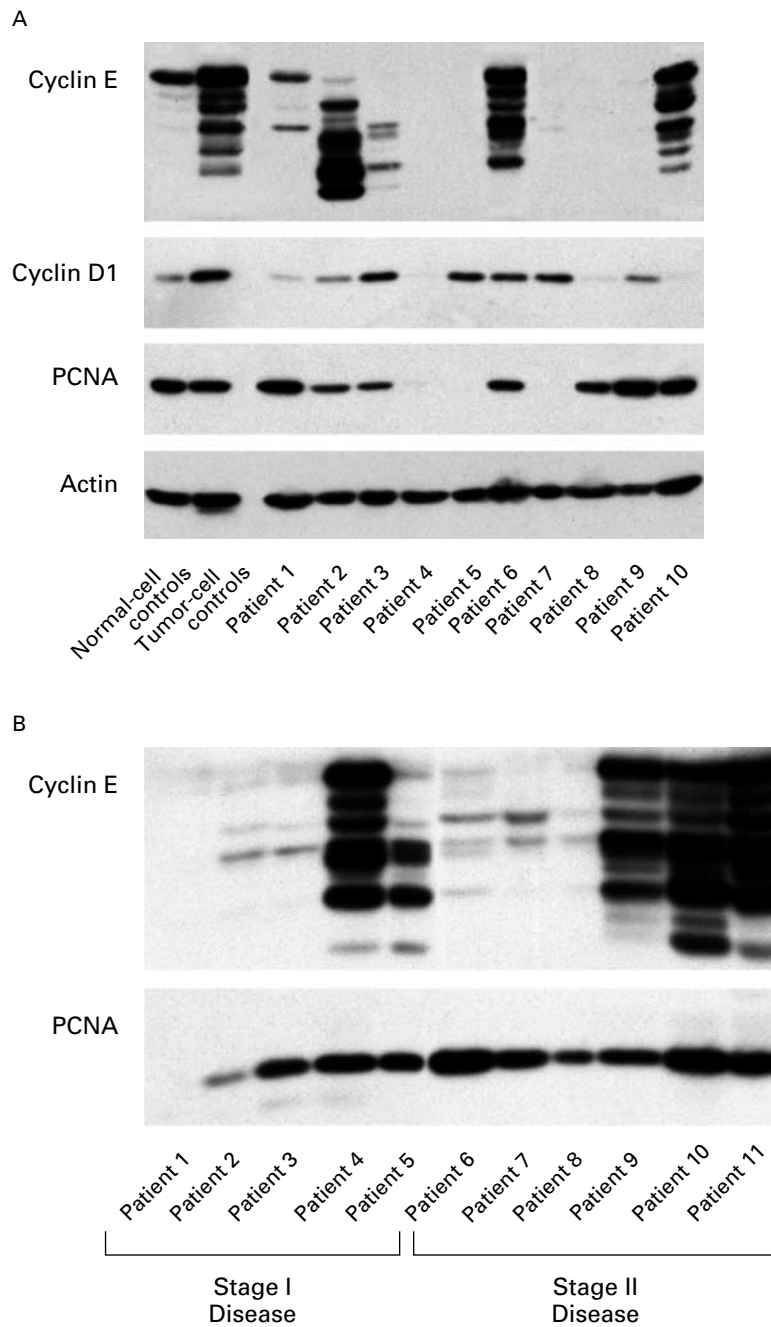
CYCLIN E AND BREAST CANCER

TABLE 1. RESULTS OF UNIVARIATE ANALYSIS OF PROGNOSTIC FACTORS.\*

FACTOR	NO. OF PATIENTS†	5-YR DISEASE-SPECIFIC SURVIVAL		5-YR OVERALL SURVIVAL	
		% (95% CI)	P VALUE	% (95% CI)	P VALUE
Age			0.74		<0.001
<50 yr	92	69 (58–77)		67 (56–76)	
≥50 yr	296	71 (66–76)		70 (65–75)	
Size of tumor			<0.001		<0.001
T1	147	87 (80–92)		81 (74–87)	
T2	169	75 (68–81)		69 (61–75)	
T3	45	41 (27–56)		39 (24–53)	
T4	33	24 (11–40)		24 (11–40)	
Nodal status			<0.001		<0.001
N0	228	86 (81–90)		81 (75–85)	
N1	139	54 (45–62)		49 (40–57)	
N2 or N3	28	34 (17–51)		32 (16–49)	
Stage of disease			<0.001		<0.001
I	114	91 (84–95)		85 (77–90)	
IIA	125	83 (75–88)		78 (70–84)	
IIB	66	70 (58–80)		63 (50–73)	
IIIA	29	50 (31–67)		44 (26–61)	
IIIB	28	29 (14–46)		29 (14–46)	
IV	33	15 (5–29)		15 (5–29)	
Estrogen-receptor status			<0.001		<0.001
Positive	234	78 (71–82)		71 (64–76)	
Negative	156	61 (53–65)		56 (48–63)	
Progesterone-receptor status			<0.001		<0.001
Positive	200	83 (77–87)		76 (69–81)	
Negative	190	59 (51–65)		56 (48–63)	
Ploidy			0.02		0.02
Diploid	112	81 (72–87)		76 (66–83)	
Aneuploid	249	67 (60–72)		62 (55–68)	
Proliferation index			<0.001		<0.001
Negative	141	85 (78–90)		79 (71–85)	
Low	83	65 (54–74)		61 (50–71)	
High	171	63 (55–70)		58 (50–65)	
HER-2/ <i>neu</i> level			<0.001		<0.001
Negative	233	82 (78–86)		77 (71–82)	
Low	29	72 (51–85)		68 (47–82)	
High	117	51 (41–60)		46 (37–55)	
Cyclin D1			<0.001		<0.001
Negative	192	79 (72–84)		73 (66–78)	
Positive	194	63 (56–70)		59 (52–66)	
Cyclin D3 level			<0.01		0.02
Negative	209	76 (70–82)		72 (65–77)	
Low	63	71 (57–80)		61 (48–72)	
High	115	62 (52–70)		59 (49–67)	
Low-molecular-weight cyclin E level			<0.001		<0.001
Low	289	91 (87–94)		84 (80–88)	
High	106	17 (10–25)		17 (10–25)	
Full-length cyclin E level			<0.001		<0.001
Low	270	93 (89–95)		86 (81–89)	
High	125	25 (17–33)		24 (17–33)	
Total cyclin E level			<0.001		<0.001
Low	268	95 (91–97)		87 (83–91)	
High	127	22 (15–30)		22 (15–30)	
Cyclin E level, according to immunohistochemical analysis			<0.001		<0.001
Low	120	81 (72–87)		75 (67–82)	
High	136	59 (50–67)		56 (47–64)	

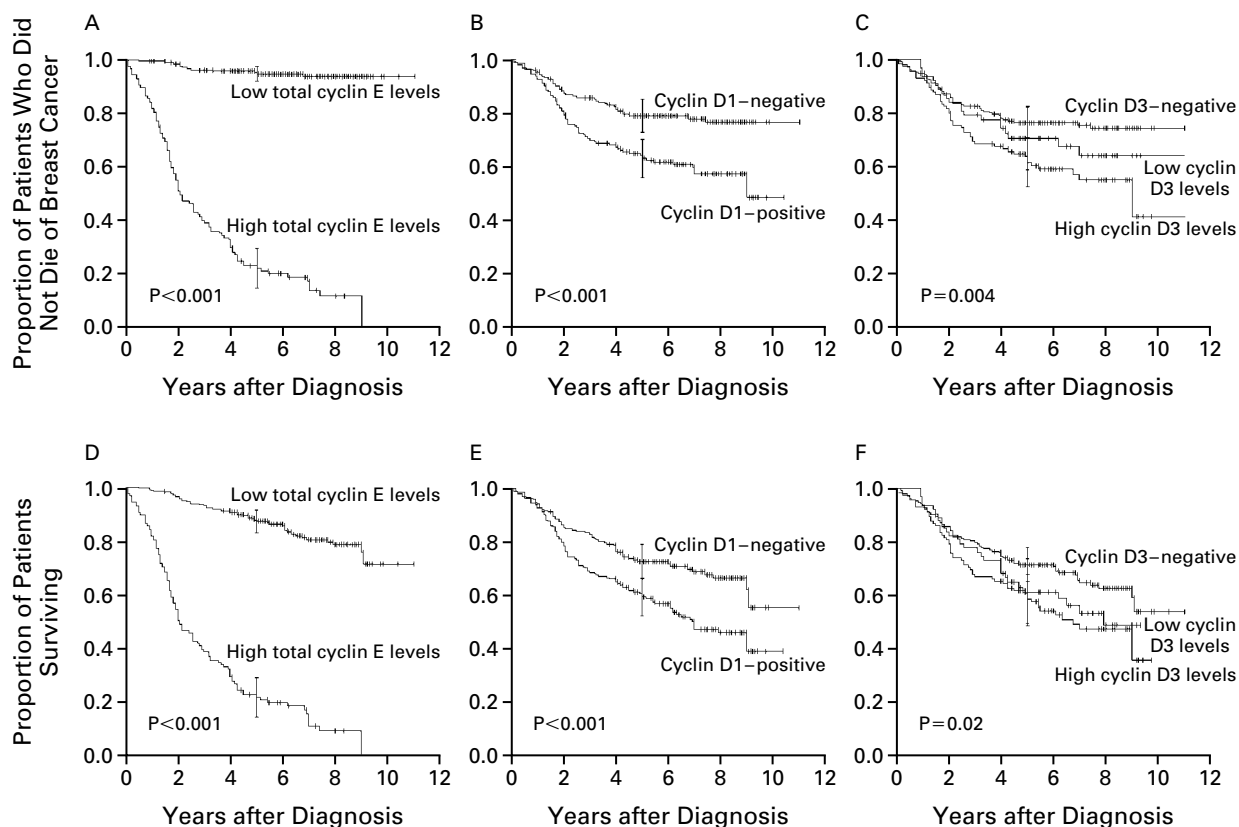
\*P values for each variable were calculated by the log-rank test. CI denotes confidence interval.

†Analyses were limited to patients for whom data were available.



**Figure 1.** Western Blots of Tumor Tissue.

In Panel A, whole-cell lysates were extracted from 10 samples of infiltrating ductal carcinoma. Patient 1 had stage I disease and had no evidence of disease at last follow-up; Patient 2 had stage IIB disease and died; Patient 3 had stage IIA disease and had no evidence of disease at last follow-up; Patient 4 had stage IIA disease and had no evidence of disease at last follow-up; Patient 5 had stage I disease and had no evidence of disease at last follow-up; Patient 6 had stage IIIB disease and died; Patient 7 had stage I disease and had no evidence of disease at last follow-up; Patient 8 had stage IIA disease and had no evidence of disease at last follow-up; Patient 9 had stage IIB disease and had no evidence of disease at last follow-up; and Patient 10 had stage IIB disease and died. Each lane contained 50  $\mu$ g of protein extract and was incubated with the indicated antibody. The control lanes represent a cultured normal mammary epithelial cell line (76N) and a cultured breast-cancer cell line (MDA-MB-157). PCNA denotes proliferating-cell nuclear antigen. In Panel B, whole-cell lysates were extracted from breast-cancer tissues from 11 patients with stage I or stage II breast cancer. Each lane contained 50  $\mu$ g of protein extract and was incubated with the indicated antibody. Patient 1 died of other causes 23 months after diagnosis. Patients 2, 3, 6, 7, and 8 were alive without evidence of disease 99 to 108 months after diagnosis, whereas Patients 4, 5, 9, 10, and 11 died of breast cancer 33 to 65 months after diagnosis.



**Figure 2.** Kaplan–Meier Estimates of Disease-Specific Survival (Panels A, B, and C) and Overall Survival (Panels D, E, and F) for All 395 Patients.

Patients are grouped according to high or low total cyclin E levels (Panels A and D), and the presence or absence of cyclin D1 (Panels B and E) or cyclin D3 (Panels C and F), as determined by Western blot analysis. The numbers of patients at risk in each group are shown in Table 1. I bars represent the 95 percent confidence intervals.

hazards model fitted to the data from the 256 patients with immunohistochemical data, only the associations with the total cyclin E level and the low-molecular-weight cyclin E level as measured by Western blot analysis retained statistical significance. The associations with the levels of total cyclin E and low-molecular-weight cyclin E, but not the association with the level of full-length cyclin E, retained significance when a model containing these factors was fitted to the data from all 395 patients.

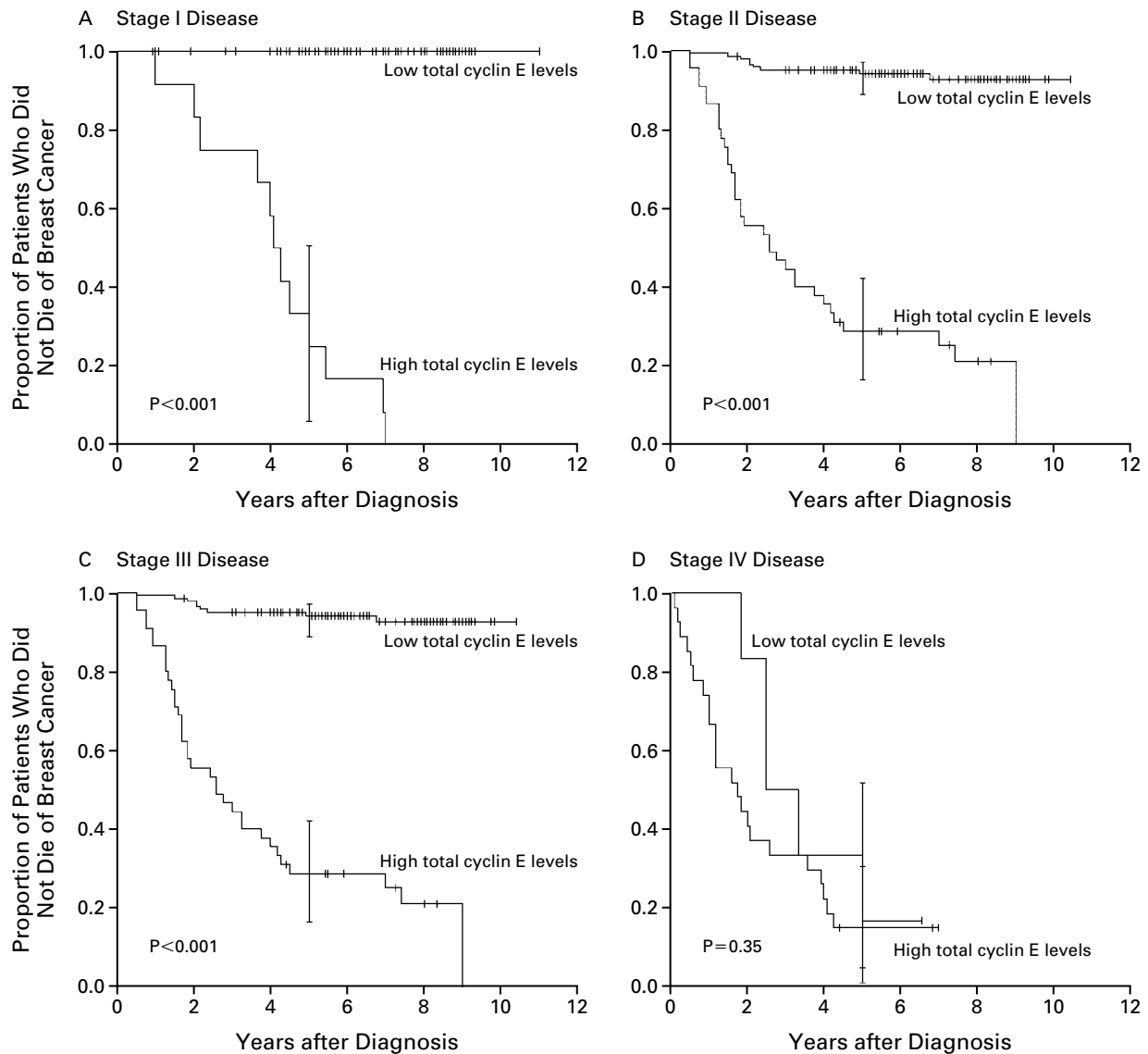
#### Immunohistochemical Analysis versus Western Blot Analysis

We next compared immunohistochemical analysis with Western blotting for measurement of cyclin E. Figures 4A and 4D show disease-specific survival and overall survival, respectively, for patients with low or high values of cyclin E as measured by immunohistochemical analysis. In Figures 4B and 4E, disease-specific survival and overall survival, respectively, are plotted for patients with high or low levels of cyclin E as

measured by Western blotting in the subgroup of patients who had low levels of cyclin E as measured by immunohistochemical analysis. Among 120 patients with low cyclin E levels as scored by immunohistochemical analysis, we identified 21 with elevated levels of cyclin E on Western blot assays. The five-year disease-specific survival rate among these patients was 19 percent (95 percent confidence interval, 5 to 39). Conversely, of 136 patients with high scores for cyclin E as judged by immunohistochemical analysis, 74 had low cyclin E levels as measured by Western blot assay. These 74 patients had a five-year disease-specific survival rate of 91 percent (95 percent confidence interval, 82 to 96) (Fig. 4C and 4F).

#### Multivariate Analysis

The results of the multivariate analysis of factors predictive of disease-specific survival and overall survival are presented in Table 2. For these analyses, all factors shown in Table 1 were initially included in the model as potential risk factors. Factors for which



**Figure 3.** Kaplan–Meier Estimates of Disease-Specific Survival According to Total Cyclin E Expression, as Measured by Western Blot Analysis.

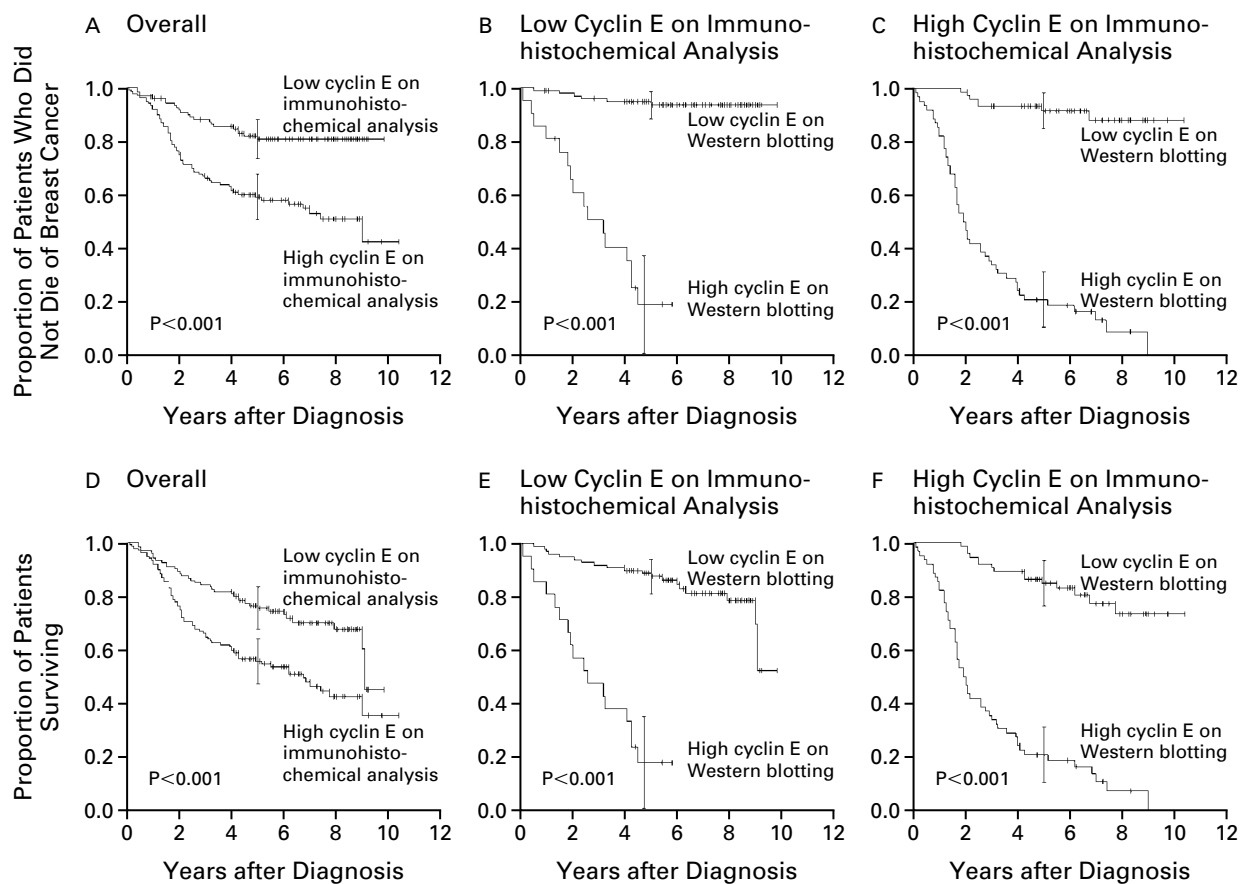
The numbers of patients at risk in each group are shown in Table 1. I bars represent the 95 percent confidence intervals.

there were missing data (e.g., cyclin E level as measured by immunohistochemical analysis and ploidy) and that were not selected by the stepwise procedure were subsequently omitted, and the stepwise procedure was repeated in order to obtain the final model. The forward and backward stepwise procedures both led to the same final model, shown in Table 2. A high total cyclin E level was strongly associated with poor outcome, with a hazard ratio for death from breast cancer of 13.3. For high levels of the low-molecular-weight isoforms of cyclin E, the hazard ratio for death from breast cancer was 2.1. Although positive lymph

nodes, negative estrogen-receptor status, and late-stage disease remained significant predictors of death from breast cancer, the association with a high cyclin E level was substantially stronger. Associations of death from breast cancer with cyclin E scores according to immunohistochemical analysis and with levels of cyclin D1, cyclin D3, and HER-2/*neu* did not reach statistical significance in the multivariate analysis.

#### DISCUSSION

In this retrospective study, we examined the correlation between levels of cyclin E and its low-molec-



**Figure 4.** Kaplan-Meier Estimates of Disease-Specific Survival and Overall Survival among Patients with Immunohistochemical Data on Cyclin E.

Survival among all 256 patients with data is shown in Panels A and D. The same patients are also grouped according to cyclin E levels (high or low), as measured by Western blotting. Panel B shows estimates of disease-specific survival and Panel E estimates of overall survival as a function of total cyclin E levels as determined by Western blot analysis in the subgroup of patients with low cyclin E levels as measured by immunohistochemical analysis. Panel C shows estimates of disease-specific survival and Panel F estimates of overall survival as a function of total cyclin E levels as determined by Western blot analysis in the subgroup of patients with high cyclin E levels as measured by immunohistochemical analysis. The numbers of patients at risk in each group are shown in Table 1. I bars represent the 95 percent confidence intervals.

**TABLE 2.** INDEPENDENT FACTORS PREDICTIVE OF DEATH FROM BREAST CANCER AND DEATH FROM ANY CAUSE.\*

FACTOR	DEATH FROM BREAST CANCER		DEATH FROM ANY CAUSE	
	HAZARD RATIO (95% CI)	P VALUE	HAZARD RATIO (95% CI)	P VALUE
High level of low-molecular-weight cyclin E	2.1 (1.1–4.0)	0.02	2.2 (1.2–4.2)	0.01
High total cyclin E level	13.3 (5.8–30.2)	<0.001	4.3 (2.2–8.4)	<0.001
Positive nodes	1.8 (1.2–2.8)	0.007	1.5 (1.1–2.2)	0.02
Stage IIIB–IV disease	1.7 (1.1–2.5)	0.01	1.7 (1.2–2.5)	0.004
Negative estrogen-receptor status	1.8 (1.3–2.7)	0.001	1.6 (1.1–2.2)	0.006

\*P values were derived from the Cox proportional-hazards model, with simultaneous inclusion of all factors shown. CI denotes confidence interval.

ular-weight forms in breast-cancer tissue and survival in patients with breast cancer. We found that the hazard ratio for death due to breast cancer in patients with high levels of total cyclin E in the tumor was higher than the hazard ratios associated with any other biologic marker we examined; it was more than seven times as high as the hazard ratio associated with lymph-node metastases. We also found that estrogen- and progesterone-receptor status and levels of HER-2/*neu*, cyclin D1, and cyclin D3 significantly correlated with disease-specific survival, but in a multivariate analysis, the cyclin E level was most closely associated with outcome. All patients with stage I disease and a high cyclin E level as determined by Western blot analysis died of breast cancer.

Previous investigations of the prognostic value of cyclin E levels in breast-cancer tissue have produced conflicting data. These studies used immunohistochemical techniques,<sup>17,18</sup> but tumor cells often overexpress low-molecular-weight forms of cyclin E<sup>7</sup> that lack the amino terminal targeted by the antibodies used in most immunohistochemical assays. In our immunohistochemical analysis of cyclin E, we used an antibody against the C terminal of the protein that recognizes both the full-length and the low-molecular-weight forms of cyclin E that are detectable by Western blot analysis.<sup>5</sup> However, we found discordance in 37 percent of the samples between the results of immunohistochemical analysis and those of Western blot assays, even though the two antibodies used in these assays targeted the same epitope of cyclin E. Since both antibodies target the same region of the protein, the reason for the difference in the ability of immunohistochemical analysis and Western blotting to assess cyclin E status reliably is not entirely clear.

The prognostic significance of cyclin E may be the result of some of its biologic functions. The low-molecular-weight forms of cyclin E are constitutively expressed in breast cancer and facilitate the transition from the G<sub>1</sub> phase to the S phase more effectively than the full-length form of the protein.<sup>8,16</sup> Constitutive overexpression of cyclin E (but not cyclin D1 or cyclin A) in immortalized rat-embryo fibroblasts and human breast epithelial cells has been shown to cause chromosomal instability.<sup>32</sup> In about 10 percent of transgenic mice that express human cyclin E, mammary carcinoma develops, demonstrating that cyclin E has oncogenic potential.<sup>33</sup> Finally, elastase, which mediates cleavage of cyclin E into its low-molecular-weight isoforms,<sup>16</sup> has also been implicated in tumor invasion and development of the metastatic phenotype.<sup>34</sup> This spectrum of biologic activity suggests that cyclin E may have multiple roles in the development and outcome of breast cancer.

In summary, in this retrospective analysis, we found high levels of low-molecular-weight and total cyclin E,

as measured by Western blotting, to be sensitive and specific prognostic indicators in patients with breast cancer. Our results are encouraging, but they must be validated in a prospective trial before they can be applied clinically. The development of molecular staging of breast cancer may have important implications for treatment, particularly in patients with early-stage disease, many of whom currently receive toxic systemic treatment with little benefit.

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Dr. Keyomarsi holds U.S. patents 5,543,291 (method of detecting carcinoma) and 5,763,219 (cyclin E variants and use thereof). Dr. Hortobagyi reports receiving research support from Ribozyme Pharmaceuticals.

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## REFERENCES

1. Voogd AC, Nielsen M, Peterse JL, et al. Differences in risk factors for local and distant recurrence after breast-conserving therapy or mastectomy for stage I and II breast cancer: pooled results of two large European randomized trials. *J Clin Oncol* 2001;19:1688-97. [Erratum, *J Clin Oncol* 2001;19:2583.]
2. Early Breast Cancer Trialists' Collaborative Group. Polychemotherapy for early breast cancer: an overview of the randomised trials. *Lancet* 1998; 352:930-42.
3. *Idem*. Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 1998;351:1451-67.
4. Dou Q-P, Pardee AB, Keyomarsi K. Cyclin E — a better prognostic marker for breast cancer than cyclin D? *Nat Med* 1996;2:254.
5. Gray-Bablin J, Zalvide J, Fox MP, Knickerbocker CJ, DeCaprio JA, Keyomarsi K. Cyclin E, a redundant cyclin in breast cancer. *Proc Natl Acad Sci U S A* 1996;93:15215-20.
6. Keyomarsi K, Pardee AB. Redundant cyclin overexpression and gene amplification in breast cancer cells. *Proc Natl Acad Sci U S A* 1993;90: 1112-6.
7. Keyomarsi K, O'Leary N, Molnar G, Lees E, Fingert HJ, Pardee AB. Cyclin E, a potential prognostic marker for breast cancer. *Cancer Res* 1994; 54:380-5.
8. Keyomarsi K, Conte D Jr, Toyofuku W, Fox MP. Deregulation of cyclin E in breast cancer. *Oncogene* 1995;11:941-50.
9. Lew DJ, Dulic V, Reed SI. Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. *Cell* 1991;66:1197-206.
10. Dou Q-P, Levin AH, Zhao S, Pardee AB. Cyclin E and cyclin A as candidates for the restriction point protein. *Cancer Res* 1993;53:1493-7.
11. Ohtsubo M, Theodoras AM, Schumacher J, Roberts JM, Pagano M. Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. *Mol Cell Biol* 1995;15:2612-24.
12. Resnitzky D, Gossen M, Bujard H, Reed SI. Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system. *Mol Cell Biol* 1994;14:1669-79.

13. Buckley MF, Sweeney KJE, Hamilton JA, et al. Expression and amplification of cyclin genes in human breast cancer. *Oncogene* 1993;8:2127-33.
14. Sgambato A, Doki Y, Schieren I, Weinstein IB. Effects of cyclin E overexpression on cell growth and response to transforming growth factor  $\beta$  depend on cell context and p27Kip1 expression. *Cell Growth Differ* 1997;8:393-405.
15. Keyomarsi K, Herliczek TW. The role of cyclin E in cell proliferation, development and cancer. In: Meijer L, Guidet S, Philippe M, eds. *Progress in cell cycle research*. Vol. 3. New York: Plenum Press, 1997:171-91.
16. Porter D, Zhang N, Danes C, et al. Tumor-specific proteolytic processing of cyclin E generates hyperactive low-molecular-weight forms. *Mol Cell Biol* 2001;21:6254-69.
17. Donnellan R, Kleinschmidt I, Chetty R. Cyclin E immunoexpression in breast ductal carcinoma: pathologic correlations and prognostic implications. *Hum Pathol* 2001;32:89-94.
18. Porter PL, Malone KE, Heagerty PJ, et al. Expression of cell-cycle regulators p27Kip1 and cyclin E, alone and in combination, correlate with survival in young breast cancer patients. *Nat Med* 1997;3:222-5.
19. Nielsen NH, Arnerlov C, Emdin SO, Landberg G. Cyclin E overexpression, a negative prognostic factor in breast cancer with strong correlation to oestrogen receptor status. *Br J Cancer* 1996;74:874-80.
20. Wang A, Yoshimi N, Suzui M, Yamauchi A, Tarao M, Mori H. Different expression patterns of cyclins A, D1 and E in human colorectal cancer. *J Cancer Res Clin Oncol* 1996;122:122-6.
21. Scuderi R, Palucka KA, Pokrovskaja K, Bjorkholm M, Wiman KG, PISA P. Cyclin E overexpression in relapsed adult acute lymphoblastic leukemias of B-cell lineage. *Blood* 1996;87:3360-7. [Erratum, *Blood* 1996; 88:4083.]
22. Bacus SS, Flowers JL, Press MF, Bacus JW, McCarty KS Jr. The evaluation of estrogen receptor in primary breast carcinoma by computer-assisted image analysis. *Am J Clin Pathol* 1988;90:233-9.
23. Bacus SS, Goldschmidt R, Chin D, Moran G, Weinberg D, Bacus JW. Biological grading of breast cancer using antibodies to proliferating cells and other markers. *Am J Pathol* 1989;135:783-92.
24. Bacus SS, Ruby SG. Application of image analysis to the evaluation of cellular prognostic factors in breast carcinoma. *Pathol Annu* 1993;28:179-204.
25. Taylor SR, Titus-Ernstoff L, Stitley S. Central values and variation of measured nuclear DNA content in imprints of normal tissues determined by image analysis. *Cytometry* 1989;10:382-7.
26. Berchuck A, Boente MP, Kerns BJ, et al. Ploidy analysis of epithelial ovarian cancers using image cytometry. *Gynecol Oncol* 1992;44:61-5.
27. Harwell RM, Porter DC, Danes C, Keyomarsi K. Processing of cyclin E differs between normal and tumor breast cells. *Cancer Res* 2000;60:481-9.
28. Lees E, Faha B, Dulic V, Reed SI, Harlow E. Cyclin E/cdk2 and cyclin A/cdk2 kinases associate with p107 and E2F in a temporally distinct manner. *Genes Dev* 1992;6:1874-85.
29. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 1958;53:457-81.
30. Greenwood M. The natural duration of cancer. *Rep Public Health Med Subjects* 1926;33:1-26.
31. Cox DR. Regression models and life-tables. *J R Stat Soc [B]* 1972;34:187-220.
32. Spruck CH, Won K-A, Reed SI. Deregulated cyclin E induces chromosome instability. *Nature* 1999;401:297-300.
33. Bortner DM, Rosenberg MP. Induction of mammary gland hyperplasia and carcinomas in transgenic mice expressing human cyclin E. *Mol Cell Biol* 1997;17:453-9.
34. Yamashita JI, Ogawa M, Ikei S, et al. Production of immunoreactive polymorphonuclear leucocyte elastase in human breast cancer cells: possible role of polymorphonuclear leucocyte elastase in the progression of human breast cancer. *Br J Cancer* 1994;69:72-6.

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## CORRECTION

**Cyclin E and Survival in Patients with Breast Cancer**

Cyclin E and Survival in Patients with Breast Cancer . On page 1572, the curves in Figure 3C were incorrect. The corrected Panel C appears below.

