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Cyclin E Low-Molecular-Weight Isoform as a Predictor of Breast Cancer in Japanese Women

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Overexpression of low-molecular-weight isoforms (LMWI) of cyclin E in breast cancer cells is associated with poor prognosis and could serve a novel role in breast cancer progression. LMWI originate from proteolytic processing of cyclin E, which is deregulated and hyperactive. In this study, levels of full-form/LMWI cyclin E were determined with the use of Western blot analysis in 69 Japanese breast cancer patients. LMWI cyclin E levels were significantly correlated with known parameters such as tumor grade and estrogen/progesterone receptor expression. In multivariate analysis, patient survival was significantly correlated with tumor grade but not with either form of cyclin E. LMWI was not as strong a predictor as tumor grade in this study, whereas some cases of early relapse with LMWI overexpression and lower tumor grade were reported. Thus, LMWI might be a good complementary factor to other predictors for early relapse of breast cancer.

Key words: Cyclin E – Low-molecular-weight form – Nuclear grade – Predictor

More than 40,000 women are newly diagnosed with breast cancer each year in Japan, and the number of breast cancer patients has been increasing. In Japan, breast cancer became the most common cancer among women in 1994, and it is the most common cause of death among Japanese women between the ages of 30 and 64. About one third of breast cancer patients develop distant metastases after the initial surgical resection.[1,2]

Breast cancer research in recent decades has yielded numerous potential prognostic factors. However, the estrogen receptor (ER) and/or progesterone receptor (PgR) and the HER-2/neu protein status are the only biologic factors besides

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clinicopathologic criteria—such as axillary lymph node status, tumor size, nuclear grade, and age—that are regularly used for clinical decision making. On the other hand, sometimes the prognosis might not be predictable based on these factors alone. For instance, it is possible to have a poor prognosis even in node-negative, estrogen-sensitive, small-tumor-size breast cancers.

Cyclin E, which regulates the G1/S-phase transition of the cell cycle, plays an important role in many different cancers.[3–6] In complex with CDK2, it is responsible for cells passing the restriction point, committing the cells to another round of division. In normal cells, cyclin E accumulates at the G1/S-phase boundary and is degraded as cells progress through the S phase.[7] Cyclin E is deregulated relative to the cell cycle in many tumors,[8] including breast tumors.[9,10]

Low-molecular-weight isoforms (LMWI) of cyclin E originate from proteolytic processing of cyclin E, which is deregulated and hyperactive.[11] Recently, total and LMWI cyclin E were described as powerful predictors of poor outcome in breast cancer.[12] The prognostic power of cyclin E surpassed that of nodal involvement, which would make it the most prominent prognostic factor for breast cancer outcome.

Some studies[13,14] concur at least in part with Keyomarsi *et al* (2002), whereas others find that cyclin E either is not an independent factor, losing its prognostic value in multivariate analysis,[15,16] or is of limited value, predicting only the site of relapse.[17]

Our aim in the present study was to reevaluate the importance of cyclin E LMWI in invasive ductal breast carcinoma in Japanese women as a prognostic marker. The results were compared with known clinicopathologic prognostic factors: lymph node metastases, estrogen/progesterone receptor status, HER-2 status, and nuclear grade.

Patients and Methods

From the identified cases, 69 specimens of invasive ductal carcinoma for which all clinical and pathologic data were available were selected from the files of the Division of Breast and Endocrine Surgery, Nagasaki University School of Medicine, National Hospital Organization Saga Hospital. This study was completed during a 5-year period from 2000 to 2004.

Patients were excluded for any of these reasons: (1) required clinicopathologic information was lacking from reports or charts; (2) slides or frozen blocks were unavailable; (3) neoadjuvant chemotherapy had been given; (4) the patient was in Stage IV; (5) the patient was a male with breast cancer.

All specimens were taken from Japanese women. For all these cases, tumor-node-metastasis (TNM) status, estrogen/progesterone receptor status, and nuclear grade were determined. HER-2 status was examined with immunohistochemistry (IHC) and/or fluorescence in situ hybridization (FISH), excluding some old cases.

The procedures for steroid receptor analysis are described later. Among a total of 69 primary breast cancers, enzyme immunoassay (EIA) was inspected between 2000 and 2002. After that, immunohistochemical staining was applied between 2003 and 2004. To evaluate the staining results for each immunohistochemically stained slide, we visually estimated the percentage of tumor cells showing nuclear reactivity. Slides in which more than 10% of tumor cells were stained were regarded as positive. Staining intensity was not evaluated.

Nuclear grade analysis has been described elsewhere.[18,19] The Japanese Breast Cancer Society recommends this analysis. Although histologic grading based on modified criteria recommended by the World Health Organization (WHO) was used for some cases, its clinical significance was considered to be almost the same as that of nuclear grade.

Methods

Cell lines

In this study, we used human anaplastic thyroid carcinoma cell line FRO as a negative control and metastatic breast cancer cell line MDA-MB-231 as a positive control.

Preparation of cell lysates

Frozen tissues were cut into very small pieces using a clean razor blade. Cell lysates were prepared by the addition of 1 volume of CelLytic MT (Sigma-Aldrich, St Louis, Missouri) (1 g tissue/20 g CelLytic MT) and protease inhibitor cocktail (Sigma-Aldrich) to crushed sample tissue and homogenation on ice. Homogenates were centrifuged at 100,000*g* for 5 minutes at 4°C, twice. Aliquots of the supernatants were then subjected to Western blot analysis.

Western blot analysis

Levels of full-length cyclin E and its LMWI were evaluated by Western blot analysis of lysates

prepared from specimens of frozen tumor tissue. Briefly, after the protein concentration had been measured by the Bradford assay, 30 μg of protein was subjected to electrophoresis on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and was transferred to a nitrocellulose membrane for 1 hour at room temperature at a constant voltage of 50 mV. The blots were blocked overnight at 4°C in blocking buffer [5% nonfat dried milk in 20 mM Tris, 137 mM NaCl, and 0.05% Tween (pH 7.6)]. After washing twice in TBST [20 mM Tris, 137 mM NaCl, and 0.05% Tween (pH 7.6)], the blots were incubated in primary antibodies overnight. The primary antibody used was cyclin E (HE-12; Santa Cruz Biotechnology, Santa Cruz, California). The blots were incubated with goat antimouse immunoglobulin-horseradish peroxidase conjugate at a dilution of 1:5000 in blocking solution for 1 hour and were finally washed. Detection was performed with an enhanced chemiluminescence kit (Amersham Life Sciences, Little Chalfont, United Kingdom).

Amounts of full-length cyclin E and low-molecular-weight cyclin E were visually assessed as negative (not visible or less than or equal to the level of protein found in negative control) or positive (visible and higher than in the negative cell control).

Statistical analyses

Associations between the expression of cyclin E full form and LMWI and other factors were examined. The Wilcoxon test was used for age and ordered categorical factors such as disease stage. Fisher's exact test was used for dichotomous factors such as lymph node metastases.

Survival rates were calculated for overall survival (OS) and disease-free survival (DFS) by the Kaplan-Meier method. Univariate Cox regression was performed for OS and DFS with 10 explanatory variables, including the status of cyclin E full form and LMWI expression. Multivariate Cox regression was carried out with only significant variables in the univariate model. *P* values less than 0.05 were considered statistically significant. SAS version 9.1.3 (SAS Institute, Cary, North Carolina) was used for all statistical analyses.

Results

Table 1 shows the characteristics of patients recruited in this study. Cyclin E full form was found in 24 patients (34.8%) and was not found in 45 (65.2%). The LMWI of cyclin E was expressed in 42 cases (60.9%) and was not expressed in 27 (39.1%). It was specifically detected in tumor tissue but not in normal tissue or in other cancer cell lines such as a thyroid papillary cancer cell line, FRO.

Table 1 Patient characteristics

Factor	Category	No. of patients
No. of patients		69 (100.0%)
Age, y	Mean	55.6
	Standard deviation	11.9
	Median	54.0
	Minimum	34
	Maximum	87
	N	69
Nodal status	Negative	44 (63.8%)
	Positive	25 (36.2%)
Nuclear grade	1	13 (18.8%)
	2	36 (52.2%)
	3	20 (29.0%)
ER	−	23 (33.3%)
	+	46 (66.7%)
PgR	−	28 (40.6%)
	+	41 (59.4%)
T (Size of tumor)	1	26 (37.7%)
	2	33 (47.8%)
	3	7 (10.1%)
	Unknown	3 (4.3%)
Stage of disease	1	21 (30.4%)
	2A	24 (34.8%)
	2B	9 (13.0%)
	3A	7 (10.1%)
	3B	5 (7.2%)
	4	0 (0.0%)
	Unknown	3 (4.3%)
HER-2	0	17 (24.6%)
	1	16 (23.2%)
	2	9 (13.0%)
	3	8 (11.6%)
	Unknown	19 (27.5%)
Cyclin E LMWF	−	42 (60.9%)
	+	27 (39.1%)
Cyclin E full form	−	24 (34.8%)
	+	45 (65.2%)
Survival	Survived	60 (87.0%)
	Dead	9 (13.0%)
Relapse	Negative	57 (82.6%)
	Positive	12 (17.4%)

The correlations between cyclin E LMWI/full-form status and known prognostic factors are displayed in Table 2. LMWI overexpression was significantly correlated with higher tumor grade and with ER/PgR status, whereas the full form showed no correlation with any of these factors.

Univariate analyses exhibited longer OS in patients younger than 55 years, those with no lymph node metastasis, those with tumor grade 1/2, and

Table 2 Cyclin E LMWI/full form and other factors

		Cyclin E LMWF		Cyclin E full form	
	Category	−	+	−	+
No. of patients		42 (100.0%)	27 (100.0%)	24 (100.0%)	45 (100.0%)
Age, y	Mean	57.4	52.7	57.0	54.8
	Standard deviation	11.9	11.7	14.8	10.2
	Median	54.0	55.0	54.0	54.0
	Minimum	37	34	34	35
	Maximum	87	72	87	79
	N	42	27	24	45
	Wilcoxon test	$P = 0.203$		$P = 0.605$	
Lymph node metastasis	Negative	29 (69.0%)	15 (55.6%)	16 (66.7%)	28 (62.2%)
	Positive	13 (31.0%)	12 (44.4%)	8 (33.3%)	17 (37.8%)
	Fisher exact test	$P = 0.309$		$P = 0.796$	
Nuclear grade	1	13 (31.0%)	0 (0.0%)	5 (20.8%)	8 (17.8%)
	2	23 (54.8%)	13 (48.1%)	15 (62.5%)	21 (46.7%)
	3	6 (14.3%)	14 (51.9%)	4 (16.7%)	16 (35.6%)
	Wilcoxon test	$P < 0.001$		$P = 0.193$	
ER	−	7 (16.7%)	16 (59.3%)	8 (33.3%)	15 (33.3%)
	+	35 (83.3%)	11 (40.7%)	16 (66.7%)	30 (66.7%)
	Fisher exact test	$P < 0.001$		$P = 1.000$	
PgR	−	10 (23.8%)	18 (66.7%)	11 (45.8%)	17 (37.8%)
	+	32 (76.2%)	9 (33.3%)	13 (54.2%)	28 (62.2%)
	Fisher exact test	$P < 0.001$		$P = 0.609$	
T (size of tumor)	1	17 (40.5%)	9 (33.3%)	9 (37.5%)	17 (37.8%)
	2	19 (45.2%)	14 (51.9%)	12 (50.0%)	21 (46.7%)
	3	4 (9.5%)	3 (11.1%)	1 (4.2%)	6 (13.3%)
	Unknown	2 (4.8%)	1 (3.7%)	2 (8.3%)	1 (2.2%)
	Wilcoxon test (exist unknown)	$P = 0.551$		$P = 0.587$	
Stage of disease	1	15 (35.7%)	6 (22.2%)	8 (33.3%)	13 (28.9%)
	2A	14 (33.3%)	10 (37.0%)	10 (41.7%)	14 (31.1%)
	2B	6 (14.3%)	3 (11.1%)	2 (8.3%)	7 (15.6%)
	3A	3 (7.1%)	4 (14.8%)	2 (8.3%)	5 (11.1%)
	3B	2 (4.8%)	3 (11.1%)	0 (0.0%)	5 (11.1%)
	4	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	Unknown	2 (4.8%)	1 (3.7%)	2 (8.3%)	1 (2.2%)
	Wilcoxon test (exist unknown)	$P = 0.156$		$P = 0.166$	
HER-2	0	11 (26.2%)	6 (22.2%)	5 (20.8%)	12 (26.7%)
	1	11 (26.2%)	5 (18.5%)	6 (25.0%)	10 (22.2%)
	2	7 (16.7%)	2 (7.4%)	3 (12.5%)	6 (13.3%)
	3	3 (7.1%)	5 (18.5%)	2 (8.3%)	6 (13.3%)
	Unknown	10 (23.8%)	9 (33.3%)	8 (33.3%)	11 (24.4%)
	Wilcoxon test (exist unknown)	$P = 0.520$		$P = 0.957$	

those with Stage I or IIA/B disease. Patients with no lymph node metastasis, tumor grade 1/2, or Stage I or IIA/B had significantly longer DFS. However, LMWI/full-form cyclin E expression was not correlated with OS or DFS (Tables 3 and 4).

Although no statistically significant difference was seen, some recurrent cases of node-negative patients showed high levels of cyclin E. For instance, patient 7 in Fig. 1 shows positive LMWI bands during 37 to 50 kD. Although she was at stage I and was estrogen/progesterone receptor-positive and HER-2 negative, she died within 4 years after diagnosis. In multivariate analyses, only tumor grade 1/2 was significantly correlated with longer OS, and Stage 1 or 2A/B was correlated with longer DFS (Tables 3 and 4, multivariate analysis). Disease free survival curves by Kaplan-Meier were shown in Figs. 2, 3, and 4. Patients with over expression for both LMWI and full forms of cyclin E had a tendency to unfavorable prognosis. Patients with Grade 3 had a poorer prognosis than Grades 1 and 2, statistically.

Table 3 OS models

				Univariate models			Multivariate models ($P < 0.05$ in univariate models)		
	Category	Number	5 yr OS (1 yr = 365.25 days)	Hazard ratio	95% CI	*P* value	Hazard ratio	95% CI	*P* value
No. of patients		69	87.9%	-	-	-	-	-	-
Age, y	>55	35	79.3%	Reference			Reference		
	≤55	34	96.9%	0.118	(0.015, 0.946)	0.044*	0.126	(0.012, 1.327)	0.085
Nodal status	Negative	44	95.2%	Reference			Reference		
	Positive	25	75.8%	6.375	(1.324, 30.697)	0.021*	1.113	(0.122, 10.116)	0.924
Nuclear grade	1 or 2	49	95.7%	Reference			Reference		
	3	20	70.0%	5.275	(1.319, 21.103)	0.019*	5.782	(1.176, 28.438)	0.031*
ER	−	23	82.6%	Reference					
	+	46	90.5%	0.415	(0.111, 1.550)	0.191			
PgR	−	28	85.7%	Reference					
	+	41	89.3%	0.578	(0.155, 2.160)	0.415			
T (size of tumor)	1	26	92.0%	Reference					
	2 or 3	40	84.7%	2.096	(0.434, 10.114)	0.357			
	Unknown	3	100.0%	<0.001	(<0.001, -)	0.994			
Stage of disease	1 or 2A/B	54	91.9%	Reference			Reference		
	3A/B or 4	12	66.7%	6.342	(1.700, 23.659)	0.006**	2.365	(0.461, 12.135)	0.302
	Unknown	3	100.0%	<0.001	(<0.001, -)	0.994	<0.001	(<0.001, -)	0.995
HER-2	0	17	86.7%	Reference					
	1	16	100.0%	<0.001	(<0.001, -)	0.995			
	2 or 3	17	76.0%	2.412	(0.467, 12.449)	0.293			
	Unknown	19	89.5%	0.733	(0.103, 5.232)	0.756			
Cyclin E LMWF	−	42	92.0%	Reference					
	+	27	81.5%	1.911	(0.511, 7.152)	0.336			
Cyclin E full form	−	24	91.5%	Reference					
	+	45	85.9%	1.129	(0.281, 4.540)	0.864			

*= $p < 0.05$.

**= $p < 0.01$.

***= $p < 0.001$.

Discussion

The need for tailor-made therapy has been discussed because of various characteristics of breast cancer. Although various markers have been discovered, the significant correlation between cyclin E LMWI and poor prognosis has been impressive.[12] Some reports have shown the usefulness of cyclin E as a prognostic factor in breast cancer only by IHC analysis.[15,17,20] The authors of those reports insisted that cyclin E might be an even more reliable marker of proliferation in node-negative patients. It has been suggested that both immunohistochemical staining and Western blot analysis of cyclin E may be beneficial in refining prognoses. Discordance in the prognostic value of cyclin E between IHC and Western blot analysis was observed in 37% of cases.[12]

We tried to confirm the prognostic usefulness of LMWI cyclin E in Japanese patients with breast cancer, but we could find no significant prognostic correlations between LMWI, cyclin E, and OS/DFS. Perhaps one reason for this is that our scoring method for validation was different from those used in other studies. Keyomarsi *et al* measured LMWI cyclin E quantitatively by densitometric scanning of corresponding bands with the use of IPLab Gel software (Molecular Dynamics, Sunnyvale, California); densitometric values for these proteins were clustered into 3 groups and were scored as negative, low level, or high level. On the other hand, our method used visual judgment of positive or negative. This method was thought insufficient for quantitative evaluation.

Other studies have also found cyclin E to be a poor prognostic marker. Berglund *et al* stated that cyclin E overexpression decreases the mobility and invasiveness of breast cancer cells.[21,22] In addition, others found that cyclin E is not an independent factor, loses its prognostic value in multivariate analysis,[15,16] or is of limited value, predicting only the site of relapse.[17]

Table 4 DFS models

				Univariate models			Multivariate models		
	Category	Number	5 yr DFS 1 y = 365.25 d)	Hazard ratio	95% CI	*P* value	Hazard ratio	95% CI	*P* value
No. of patients		69	83.3%	-	-	-	-	-	-
Age, y	>55	35	76.2%	Reference					
	≤55	34	90.4%	0.306	(0.083, 1.130)	0.076			
Nodal status	Negative	44	93.0%	Reference			Reference		
	Positive	25	67.3%	5.799	(1.568, 21.441)	0.008**	2.186	(0.424, 11.263)	0.350
Nuclear grade	1 or 2	49	88.9%	Reference			Reference		
	3	20	70.0%	3.880	(1.231, 12.236)	0.021*	2.539	(0.770, 8.368)	0.126
ER	−	23	69.3%	Reference					
	+	46	90.4%	0.318	(0.101, 1.001)	0.050			
PgR	−	28	74.6%	Reference					
	+	41	89.2%	0.453	(0.144, 1.429)	0.177			
T (size of tumor)	1	26	88.1%	Reference					
	2 or 3	40	79.3%	1.875	(0.507, 6.931)	0.346			
	Unknown	3	100.0%	<0.001	(<0.001, -)	0.993			
Stage of disease	1 or 2A/B	54	92.4%	Reference			Reference		
	3A/B or 4	12	41.7%	7.827	(2.474, 24.768)	<0.001***	4.165	(1.006, 17.255)	0.049*
	Unknown	3	100.0%	<0.001	(<0.001, -)	0.995	<0.001	(<0.001, -)	0.994
HER-2	0	17	86.7%	Reference					
	1	16	93.8%	0.492	(0.045, 5.432)	0.563			
	2 or 3	17	63.1%	3.744	(0.777, 18.032)	0.100			
	Unknown	19	89.5%	0.780	(0.110, 5.545)	0.804			
Cyclin E LMWF	−	42	89.4%	Reference					
	+	27	73.7%	2.357	(0.748, 7.430)	0.143			
Cyclin E full form	−	24	87.1%	Reference					
	+	45	80.9%	1.669	(0.451, 6.176)	0.443			

*= $p < 0.05$.

**= $p < 0.01$.

***= $p < 0.001$.

Cyclin E was described in a special article of the American Society of Clinical Oncology, ''2007 Update of Recommendations for the Use of Tumor Markers in Breast Cancer.''[23] Although cyclin E as a prognostic marker is promising, additional properly designed studies are required to ascertain the clinical usefulness of cyclin E.

Cyclin E has been compared with nuclear grade, but correlations between cyclin E, LMWI, and nuclear grades have not been confirmed yet. Nuclear grade is an important and indispensable factor in decision making for adjuvant therapy, which was advocated for several decades.[18,19,24–28] Those reports showed strong correlations with each other. Because mitotic count forms an integral part of the grading of breast ductal carcinoma, it is not surprising that high levels of cyclin E correlate with high grade. More mitotically active tumors also grow faster, so it could be expected that cyclin E–positive tumors are larger than their negative counterparts, as shown in our study. Keyomarsi *et al* noted in a letter[29] that data regarding the prognostic value of tumor grade in patients with small, node-negative tumors are not consistent. However, they also compared proliferation markers such as Ki-67 with cyclin E. They concluded that cyclin E, when compared with Ki-67 staining, remained the single most significant predictor of survival; it was at least 10 times more powerful than either proliferation.

High-grade tumors were also more likely to be ER-negative. Thus, grade may be a confounding variable in the correlation between cyclin E and ER. Whether cyclin E has any direct effect on ER expression remains to be determined.[30] Overall, no independent prognostic significance can be assigned to cyclin E, mainly because it is very difficult to separate the contribution of cyclin E from those of other prognostic factors, such as grade and ER status.

In conclusion, cyclin E appeared to contribute to prognosis in breast ductal carcinoma primarily

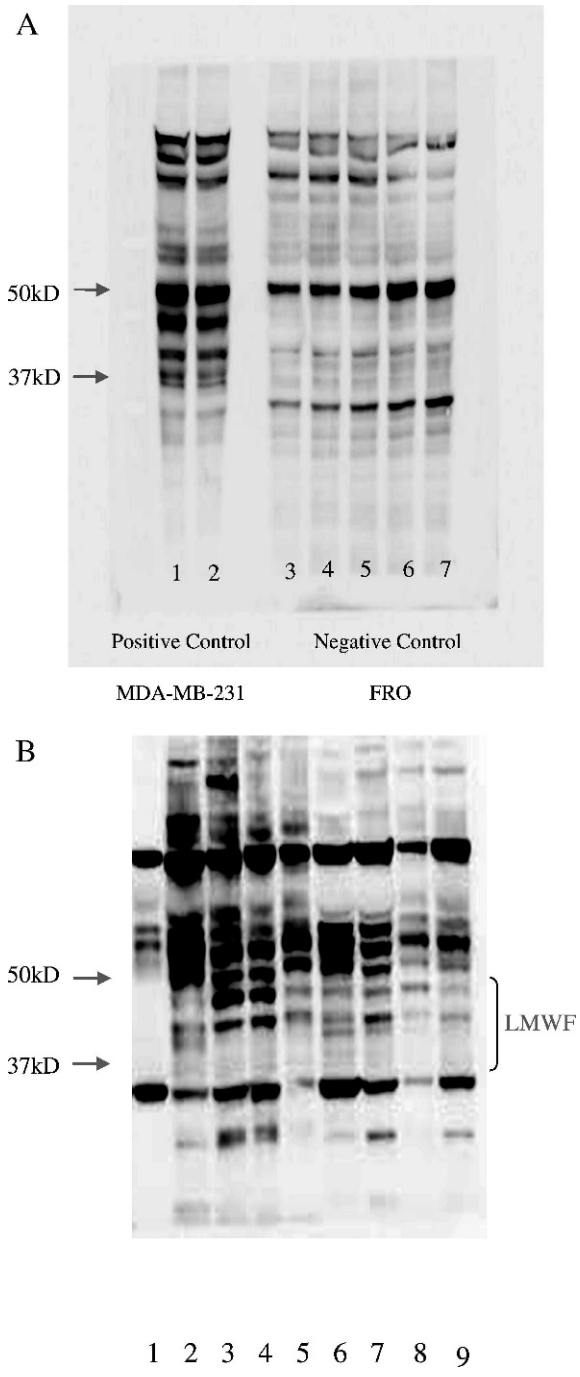

Fig. 1 Western blots of tumor tissues. In panel A, the control lanes represent a thyroid cancer cell line (FRO) and a cultured breast cancer cell line (MDA-MB-157). Each lane contains 30 μg of protein extract incubated with the indicated antibody. In Panel B, whole-cell lysates were extracted from 9 samples of ductal carcinoma and others. Patient 1 had fibroadenoma; patient 2 had stage I disease and died in 4 years; patient 3 had stage IIB disease and died in 4 years; patient 4 had stage IIIA disease and died in 3 years; patient 5 had stage I disease and had no evidence of disease at last follow-up; patient 6 had stage I disease and had no evidence of disease at last follow-up, but nuclear grade was not available and thus this patient is not included in this examination; patient 7 had stage IIB disease and showed no evidence of disease at last follow-up; patient 8 had fibroadenoma; and patient 9 had ductal carcinoma in situ. Each lane contained 30 μg of protein extract and was incubated with the indicated antibody.

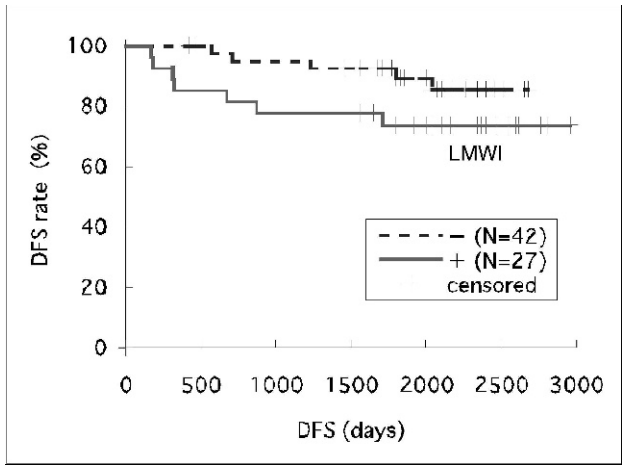

Fig. 2 DFS and cyclin E (LMWI). Log-rank test, $P = 0.131$.

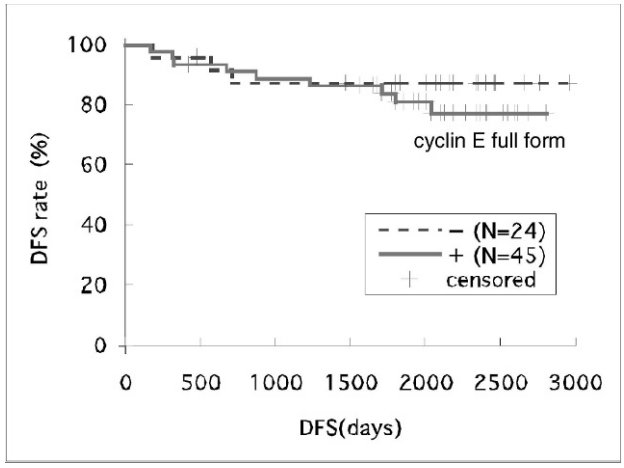

Fig. 3 DFS and cyclin E full form. Log-rank test, $P = 0.438$.

through its contribution to proliferation in Japanese women. Our results on the value of cyclin E LMWI showed that it was not more effective than known factors, especially nuclear grade. Thus, at present, evidence is insufficient to incorporate assessment of cyclin E into the workup of all Japanese patients with breast ductal carcinoma. Whether or not cyclin E analysis improves the assessment of prognosis in

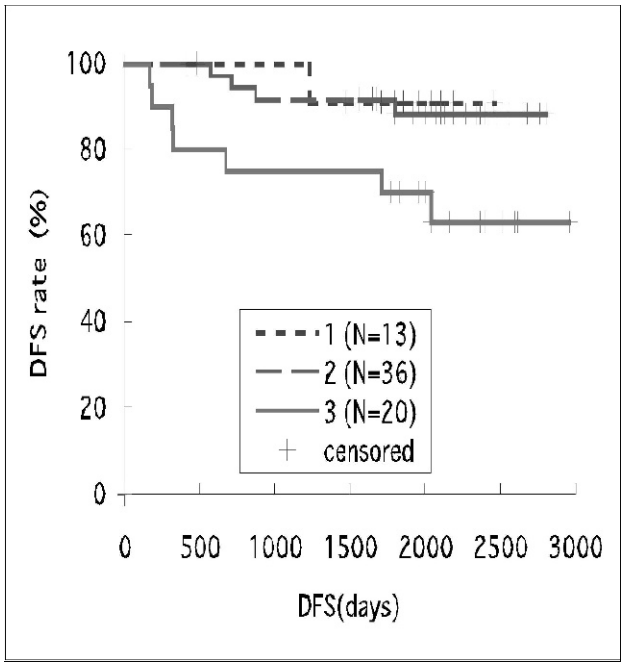

Fig. 4 DFS and nuclear grade. Log-rank test 1 versus 2, $P = 0.837$. Log-rank test 1 versus 3, $P = 0.113$. Log-rank test 2 versus 3, $P = 0.027$.

breast cancer patients will require confirmation through greater numbers and verification in other patient populations.

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