Table 3. Univariate and multivariate analysis of prognostic factors

	Univariate		Multivariate	
	HR (95% CI)	P	HR (95% CI)	P
Menopausal status	1.13 (0.71-1.79)	0.602		
Tumor size (>5 cm)	5.33 (1.76-13.4)	0.005	4.37 (1.43-11.0)	0.013
Histologic grade	1.63 (0.86-2.88)	0.130		
Lymph node status	1.01 (0.64-1.63)	0.964		
Stage	1.21 (0.89-1.64)	0.226		
Estrogen receptor status	0.72 (0.46-1.17)	0.182		
Progesterone receptor status	0.66 (0.41-1.04)	0.072		
CD55 expression	2.00 (1.23-3.32)	0.005	1.89 (1.16-3.16)	0.011

Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval.

prognosis. The present study showed that CD55-high character was a poor prognostic factor, even when the percentage of cells with significant high CD55 expression was as low as 1%. Presence of cells showing CD55 strong expression, irrespective of percentage of such cells among tumor cells, is a sign for aggressiveness of tumors.

Taken together, the presence of a small population strongly expressing CD55 was a poor prognostic factor in breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Heppner GH. Tumor heterogeneity. Cancer Res 1984; 44:2259–65.
2. Hemburger AW, Salmon SE. Primary bioassay of human tumor stem cells. J Clin Invest 1977;197:461–3.
3. Bruce WR, Van Der Gaag H. A quantitative assay for the numner of murine lymphoma cells capable of proliferation *in vivo*. Nature 1963;199:79–80.
4. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 1997;3:730–7.
5. Lessard J, Sauvageau G. Bmi-1 determined the proliferative capacity of normal and leukaemic stem cells. Nature 2003;423:255–60.
6. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 2003;100:3983–8.
7. Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumour initiating cells. Nature 2004;432: 396–401.
8. Reya T, Morrison SJ, Clarke MF, et al. Stem cells, cancer, and cancer stem cells. Nature 2001;414:105–11.
9. Collins AT, Berry PA, Hyde C, et al. Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 2005;65:10946–51.
10. O'Brien CA, Pollett A, Gallinger S, et al. A human colon cancer cell capable of initiating tumor growth in immunodeficient mice. Nature 2007;445:106–10.
11. Ricci-Vitiani L, Lombardi DG, Pilozzi E, et al. Identification and expansion of human colon-cancer-initiating cells. Nature 2007;445:111–5.
12. Li C, Heidt DG, Dalerba P, et al. Identification of pancreatic cancer stem cells. Cancer Res 2007;67: 1030–7.
13. Prince ME, Sivanandan R, Kaczorowski A, et al. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. Proc Natl Acad Sci U S A 2007;104: 973–8.
14. Kondo T, Setoguchi T, Taga T. Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. Proc Natl Acad Sci U S A 2004;101:781–6.
15. Patrawala L, Calhoun T, Schneider-Broussard R, et al. Side population is enriched in tumorigenic, stem-like cancer cells, whereas ABCG2+ and ABCG2- cancer cells are similarly tumorigenic. Cancer Res 2005;65: 6207–19.
16. Mikesch JH, Buerger H, Simon R, Brandt B. Decay-accelerating factor (CD55): a versatile acting molecule in human malignancies. Biochim Biophys Acta 2006; 1766:42–52.
17. Spendlove I, Ramage JM, Bradley R, Harris C, Durrant LG. Complement decay accelerating factor (DAF)/CD55 in cancer. Cancer Immunol Immunother 2006;55:987–95.
18. Li L, Spendlove I, Morgan J, Durrant LG. CD55 is overexpressed in the tumour environment. Br J Cancer 2001;84:80–6.
19. Nasu J, Mizuno M, Uesu T, et al. Cytokine-stimulated release of decay-accelerating factor (DAF; CD55) from HT-29 human intestinal epithelial cells. Clin Exp Immunol 1998;113:379–85.
20. Nonaka M, Miwa T, Okada N, Nonaka M, Okada H. Multiple isoforms of guinea pig decay-accelerating factor generated by alternative splicing. J Immunol 1995;155:3037–48.
21. Yu J, Caragine T, Chen S, Morgan BP, Frey AB, Tomlinson S. Protection of human breast cancer cells from complement-mediated lysis by expression of heterologous CD59. Clin Exp Immunol 1999;115:13–8.
22. Xu JX, Morii E, Liu Y, et al. High tolerance to apoptotic stimuli induced by serum depletion and ceramide in side-population cells: high expression of CD55 as a novel character for side-population. Exp Cell Res 2007;313:1877–85.
23. Loberg RD, Wojno KJ, Day LL, Pienta KJ. Analysis of membrane-bound complement regulatory proteins in prostate cancer. Urology 2005;66:1321–6.
24. Inoue T, Yamakawa M, Takahashi T. Expression of complement regulating factors in gastric cancer cells. Mol Pathol 2002;55:193–9.
25. Terui Y, Sakurai T, Mishima Y, et al. Blockage of bulky lymphoma-associated CD55 expression by RNA interference overcomes resistance to complement-dependent cytotoxicity with rituximab. Cancer Sci 2006;97:72–9.
26. Koretz K, Bruderlein S, Henne C, Moller P. Decay-accelerating factor (DAF, CD55) in normal colorectal mucosa, adenomas and carcinomas. Br J Cancer 1992;66:810–4.
27. Loberg RD, Day LL, Dunn R, Kakikin LM, Pienta KJ. Inhibition of decay-accelerating factor (CD55) attenuates prostate cancer growth and survival *in vivo*. Neoplasia 2006;8:69–78.
28. Durrant LG, Chapman MA, Buckley DJ, Spendlove I, Robins RA, Armitage NC. Enhanced expression of the complement regulatory protein CD55 predicts a poor prognosis in colorectal cancer patients. Cancer Immunol Immunother 2003;52:638–42.
29. Madjd Z, Durrant LG, Bradley R, Spendlove I, Ellis IO, Pinder SE. Loss of CD55 is associated with aggressive breast tumors. Clin Cancer Res 2004;10: 2797–803.
30. Meyer-Siegler KL, Iczknowski KA, Leng L, Bucala R, Vera PL. Inhibition of macrophage migration inhibitory factor or its receptor (CD74) attenuates growth and invasion of DU-145 prostate cancer cells. J Immunol 2006;177:8730–9.
31. Machin D, Campbell M, Fayers P, Pinol A. Comparing two survival curves. In: Macin D, Campbell M, Fayers P, Pinol A, editors. Sample size tables for clinical studies. London: Blackwell Science; 1997. p.176–7.
32. Zhang J, Hughs SE. Role of the ephrin and Eph receptor tyrosine kinase families in angiogenesis and development of the cardiovascular system. J Pathol 2006;208:453–61.
33. Zhong RK, Kozil R, Ball ED. Homologous restriction of complement-mediated cell lysis can be markedly enhanced by blocking decay-accelerating factor. Br J Haematol 1995;91:269–74.
34. Brandt B, Mikesch JH, Simon R, et al. Selective expression of splice variant of decay-accelerating factor in c-erbB-2-positive mammary carcinoma cells showing increased transendothelial invasiveness. Biochem Biophys Res Commun 2005;329:318–23.
35. Naoi Y, Miyoshi Y, Taguchi T, et al. Connexin 26 expression is associated with lymphatic vessel invasive and poor prognosis in human breast cancer. Breast Cancer Res Treat. Epub 2007 Jan 3.

original article

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Determination of the specific activity of CDK1 and CDK2 as a novel prognostic indicator for early breast cancer

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Background: We recently established a novel assay for specific activity (SA) of cyclin-dependent kinases (CDKs) using small tumor samples (≥8 mm^3). The aim of this study was to investigate the prognostic significance of CDK1SA and CDK2SA in human breast cancer.

Methods: CDK1SA and CDK2SA were determined in 284 breast cancer patients and their prognostic significance was investigated.

Results: Tumors with high CDK1SA and high CDK2SA showed significantly poorer 5-year relapse-free survival than those with low CDK1SA and low CDK2SA, respectively (66.9% vs 84.2% for CDK1SA; 43.6% vs 83.6% for CDK2SA). Moreover, combined analysis of CDK1SA and CDK2SA enabled the classification of breast tumors into high-risk and low-risk groups, where tumors in the high-risk group were strongly associated with unfavorable prognosis (5-year relapse-free survival 69.4% for the high-risk group and 91.5% for the low-risk group). Multivariate analysis showed that the risk determined by combined analysis of CDK1SA and CDK2SA is a significant (hazard ratio 3.09, $P < 0.001$) prognostic indicator for relapse, especially in node-negative patients (hazard ratio 6.73, $P < 0.001$).

Conclusion: Determination of CDK1SA and CDK2SA may be useful in the prediction of outcomes in breast cancer patients and has potential for use as a routine laboratory test.

Key words: breast cancer, cycline dependent kinase, prognosis

original article

introduction

It is well established that systemic adjuvant therapy for early breast cancer significantly reduces the risk of recurrence and death regardless of nodal status [1, 2]. However, the fact that approximately two-thirds of node-negative patients can survive without recurrence even without adjuvant therapy indicates that adjuvant therapy is administered to many patients who actually do not need it. To avoid unnecessary treatments, we need new and more powerful prognostic indicators [3, 4].

Recently, molecules involved in cell cycle regulation such as cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors have been attracting considerable attention as potential prognostic indicators [4–6]. Cyclin E appears to be the most promising of these molecules. High cyclin E expression detected by western blotting has been shown to be strongly associated with unfavorable prognosis, independent of nodal status [5]. However, it is not easy to reproducibly assay total cyclin E or low molecular weight cyclin E expression by western blotting, which does not seem to be suitable for routine laboratory tests.

We have been focusing on CDKs (CDK1 and 2) and investigating their prognostic significance in breast cancers because CDKs play a pivotal role in cell cycle regulation [7, 8]. The CDK expression levels are almost constant but their activities change markedly according to the cell cycle phase. Thus, it is necessary to measure CDK activity itself to accurately evaluate the role of CDKs in cell proliferation. Recently, we succeeded in developing a system that can assay the specific activity (SA) of CDKs using small tissue samples [9]. The aim of this study was to clarify the prognostic implications of CDKSA in breast cancers.

patients and methods

patients

For this study, 284 patients with primary invasive breast cancer who had undergone mastectomy or breast-conserving surgery between November 1996 and December 2002 were recruited. Of these 284 patients, 162 patients were given hormonal therapy (tamoxifen alone, 124; tamoxifen plus luteinizing hormone-releasing hormone analog, 31; other modalities, 7), 37 patients underwent chemotherapy (cyclophosphamide, methotrexate and 5-fluorouracil [CMF], 16; cyclophosphamide plus epirubicin [CE], 19; other modalities, 2) and 61

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patients received chemohormonal therapy (CMF plus tamoxifen, 17; CE plus tamoxifen, 25; other modalities, 19).

The median follow-up period was 56.6 (8–89) months, and the relapse-free survival rate at 5 years after surgery (5yRFS) was 80.9%. Forty-nine patients developed recurrence (liver, 6; lung, 9; bone, 11; soft tissue, 23). Ipsilateral breast recurrences after breast-conserving surgery were not counted as recurrences.

assay for CDKSA

The assay of CDKSA consists of analyses of protein expression and kinase activity, as previously described [9]. In brief, lysates of frozen tissues were prepared with a homogenizer and stored at −80 °C until use. For expression analysis, the lysate was applied to an ImmobiChip (Sysmex, Kobe, Japan). The target protein was detected by sequential reactions with primary antibodies (anti-CDK1, anti-CDK2 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH); Sysmex, Kobe, Japan), biotinylated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and fluorescein-labeled streptavidin (Vector, Burlingame, CA). For kinase activity analysis, the CDK1 or CDK2 molecules in the lysate were first captured in a mini-column coupled with anti-CDK1 or anti-CDK2 antibody. Then an in-column kinase reaction and a fluorescein labeling reaction were performed sequentially, and the final reaction mixture was applied to the ImmobiChip. For quantification of both CDK expression and activity, catalytically active recombinant CDK1 or CDK2 (Upstate Biotechnology, Lake Placid, NY) was used as a standard. The CDKSAs were then calculated as kinase activity (U/μL lysate, where 1 U is equivalent to the activity of 1 ng of standard) divided by its corresponding expression (ng/μL lysate). The cut-off values for CDK1SA, CDK2SA and CDK2SA/CDK1SA ratio were defined as the points that gave the best discrimination in RFS. The optimal cut-off points were 100 U/ng for CDK1SA, 800 U/ng for CDK2SA and 5.6 for CDK2SA/CDK1SA. The distribution of breast tumors according to CDK1SA and CDK2SA is shown in Figure 1.

assay for human epidermal growth factor receptor type 2 expression

HER2 expression was examined by HercepTest (DakoCytomation, Carpinteria, CA) in 195 patients and by western blotting in 87 patients whose primary tissues were not available for HercepTest. The insoluble membrane fraction of the lysate for CDKSA assay was solubilized by RIPA buffer-supplemented protease inhibitor cocktail (SIGMA-Aldrich, St Louis, MO). The resultant supernatant was electrophoresed followed by transfer to PVDF membrane. After blocking, the membrane was treated with polyclonal anti-HER2 antibody (Upstate Biotechnology, Lake Placid, NY), biotinylated anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and Alexa-Fluor488-streptavidin (Molecular Probes, Eugene, OR). Fluorescent signal intensities of HER2 were measured and normalized to GAPDH expression. HER2 expression was classified as negative, 1+ or 2+. A high concordance (82%) between score 3+ of HercepTest and 2+ of the western blotting was confirmed (data not shown), and both were defined as HER2-positive.

statistical methods

RFS was calculated with the Kaplan–Meier method, and the differences were assessed with the log-rank test. The Cox proportional hazards model was used for both univariate and multivariate analyses. Test results were considered significant for $P \leq 0.05$.

results

relationship of various clinicopathologic parameters or CDK1/2SA with prognosis

The relationship of various clinicopathologic parameters with 5yRFS is shown in Table 1. Lymph node metastases, high histologic grade, estrogen receptor (ER) negativity, progesterone receptor (PR) negativity and HER2 positivity were significantly associated with poor 5yRFS. With respect to

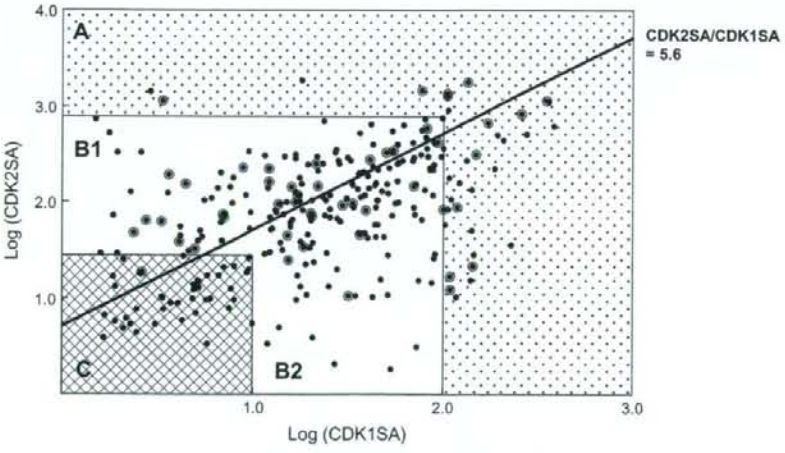

Figure 1. Distribution of breast tumors according to CDK1SA and CDK2SA. Tumors ($n = 284$) are plotted in two dimensions (logarithmic scales) according to CDK1SA and CDK2SA. Area A includes tumors with high CDK1SA (>100 U/ng) and/or high CDK2SA (>800 U/ng) ($n = 37$). Area C includes tumors where both CDK1SA and CDK2SA are less than lower measurement limits ($n = 33$). The remaining tumors are divided into two groups (B1 [$n = 85$] and B2 [$n = 129$]) according to the CDK2SA to CDK1SA ratio, with a cut-off at 5.6. Tumors in areas A and B1 are considered to be high-risk for relapse (CDK-based high-risk group) and those in areas B2 and C to be low-risk (CDK-based low-risk group). Tumor without relapse; ●, tumor with relapse; ●.

Table 1. Association between tumor parameters and 5-year RFS in all patients ($n = 284$)

Parameters	Category	No. of patients ($n = 284$)	5yRFS (%)	P value*
Age	<50 years	113	84.1	0.546
	≥50 years	171	80.2	
Tumor size	≤2.0 cm	118	85.5	0.082
	>2.0 cm	166	78.7	
Lymph node status	Negative	178	87.7	0.0006
	Positive	105	70.7	
Histologic grade	1	76	89.3	0.018
	2 + 3	206	78.6	
ER[a]	Positive	167	85.7	0.009
	Negative	111	76.2	
PR[a]	Positive	165	86.0	0.007
	Negative	113	75.7	
HER2	Negative	247	82.4	0.028
	Positive	32	62.7	
CDK1SA	Low	251	84.2	0.004
	High	33	66.9	
CDK2SA	Low	273	83.6	<0.0001
	High	11	43.6	
CDK2SA/CDK1SA ratio	Low	187	88.8	0.0001
	High	97	68.7	
CDK-based risk[b]	Low	162	91.5	<0.0001
	High	122	69.4	

*P value was evaluated by the log-rank test and was considered significant for $P \leq 0.05$.

[a]Estrogen receptor (ER) and progesterone receptor (PR) levels in tumors were measured with an enzyme immunoassay. The respective cut-off values for ER and PR were 13 and 10 fmol/mg protein.

[b]CDK-based risk was determined by the combination of CDK1SA and CDK2SA. CDK-based low-risk group was composed of patients with tumors showing both CDK1SA and CDK2SA less than lower measurement limits (area C in Figure 1) and those with a low ratio of CKD2SA/CDK1SA (area B2 in Figure 1). The CDK-based high-risk group was composed of patients with tumors showing high CDK1SA and/or high CDK2SA (area A in Figure 1) and those with a high ratio of CKD2SA/CDK1SA (area B1 in Figure 1).

HER2, Human Epidermal Growth Factor Receptor Type 2.

CDKSAs, patients with high CDK1SA and high-CDK2SA tumors showed a significantly lower 5yRFS than those with low CDK1SA and low-CDK2SA tumors, respectively. Moreover, patients with tumors with a high CDK2SA/CDK1SA ratio showed a significantly lower 5yRFS than those with tumors with a low CDK2SA/CDK1SA ratio.

Next, we studied the relationship of the combination of CDK1SA and CDK2SA with prognosis. Patients with high CDK1SA and/or high-CDK2SA tumors (area A in Figure 1) showed a poor prognosis (5yRFS rate 60%), whereas patients with tumors where both CDK1SA and CDK2SA were less than lower measurement limits (area C in Figure 1) were unlikely to develop recurrent diseases (5yRFS rate 96%). The remaining patients were able to be divided into the high- and low-risk groups according to the CKD2SA/CDK1SA ratio; that is, patients with tumors with a high CKD2SA/CDK1SA ratio (area B1 in Figure 1) were at high risk of relapse (5yRFS rate 73%) and those with a low CKD2SA/CDK1SA ratio (area B2 in Figure 1) were at low risk of relapse (5yRFS rate 91%). Accordingly, using the combination of CDK1SA and CDK2SA, all patients could be classified into a CDK-based low-risk group (area B2 and C in Figure 1) and a CDK-based high-risk group (area A and B1 in Figure 1). Patients in the CDK-based high-risk group showed a significantly lower 5yRFS than those in the CDK-based low-risk group (Table 1 and Figure 2A).

The prognostic impacts of various markers were evaluated by univariate and multivariate analyses (Table 2). In the univariate analysis, lymph node status, histologic grade, ER, PR, HER2 and CDK-based risk were significantly associated with relapse. In the multivariate analysis, however, only lymph node status and CDK-based risk had a significant correlation with relapse (hazard ratio 2.22 and 3.09, respectively).

CDK1/2SA and clinicopathologic parameters. The relationship of CDK-based risk with clinicopathologic parameters was evaluated with the chi-square test. CDK-based high risk showed a significant association with large tumor size ($P = 0.035$), lymph node involvement ($P = 0.046$), high histologic grade ($P = 0.0008$) and PR negativity ($P = 0.004$), but no significant association with ER ($P = 0.362$) and HER2 status ($P = 0.118$).

CDK1/2SA and prognosis according to nodal status. In both node-negative and node-positive subsets, patients in the CDK-based high-risk group showed a significantly lower 5yRFS than those in the CDK-based low-risk group (node-negative, 72.6% vs 97.8%; node-positive, 61.0% vs 79.0%) (Figure 2B and 2C).

In the node-positive group, univariate analysis showed that the number of metastatic lymph nodes, ER status and CDK-based risk were significantly associated with relapse, whereas multivariate analysis showed only that the number of metastatic lymph nodes and ER status were significant prognostic indicators for relapse (data not shown). In the node-negative group, univariate analysis showed that the CDK-based risk had a significant association with relapse, and that the histologic grade and PR status had a tendency to be associated with relapse. The multivariate analysis demonstrated that only CDK-based risk is a significant independent prognostic indicator (hazard ratio 6.73).

prognostic factors for node-negative patients receiving hormonal therapy alone

Of 178 node-negative patients, 139 (78%) patients received hormone therapy alone as adjuvant therapy, and 14 of these 139 patients developed recurrences. Neither histologic grade nor the St Gallen's criteria [10], widely used as the risk classification especially for node-negative patients, showed a significant association with relapse in these 139 patients (Figure 2D and 2E). However, patients in the CDK-based high-risk group showed a significantly lower

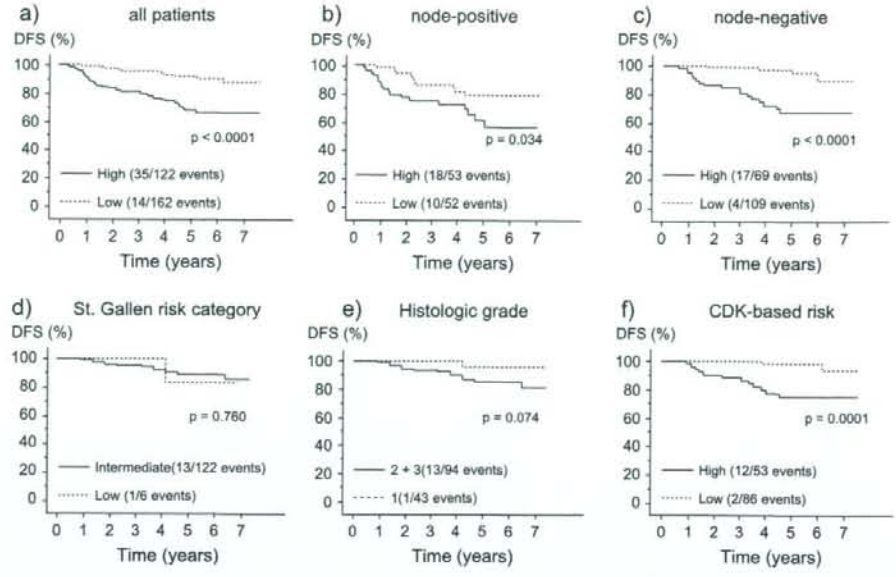

Figure 2. Relapse-free survival (RFS) rates according to the CDK-based risk and St Gallen's risk categorization. In (A) all, (B) node-positive and (C) node-negative patients, CDK-based high risk was strongly associated with poor prognosis compared to CDK-based low risk. In node-negative patients receiving hormone therapy alone as systemic adjuvant therapy, risk classification according to (D) St. Gallen risk category (2005 version) and (E) histologic grade failed to show a significant difference in RFS. (F) CDK-based risk was able to classify these patients into the high- and the low-risk groups, and their 5-year RFS rates were 74.9% vs 98.4%, respectively ($P = 0.0001$).

Table 2. Univariate and multivariate analyses for relapse in all patients ($n = 284$)

Parameter		Univariate			Multivariate		
		Hazard ratio	95% CI*	*P* value	Hazard ratio	95% CI*	*P* value
Age	< 50 vs ≥ 50 years	0.84	0.47–1.50	0.546			
Tumor size	>2.0 vs ≤2.0 cm	1.70	0.93–3.13	0.086			
Lymph node status	Positive vs negative	2.59	1.47–4.56	0.001	2.22	1.24–3.95	0.007
Histologic grade	2 + 3 vs 1	2.69	1.15–6.32	0.023	1.88	0.79–4.48	0.155
ER	Negative vs positive	2.10	1.19–3.71	0.011			
PR	Negative vs positive	2.15	1.22–3.81	0.009	1.50	0.83–2.72	0.181
HER2	Positive vs negative	2.21	1.07–4.59	0.033	1.87	0.87–3.99	0.108
CDK-based risk	High vs low	3.93	2.11–7.32	<0.0001	3.09	1.64–5.82	0.0005

*CI, confidence interval; HER2, Human Epidermal Growth Factor Receptor Type 2.

5yRFS than those in the CDK-based low-risk group (74.9% vs 98.4%, $P = 0.0001$) (Figure 2F).

discussion

In this study, we applied our novel assay system to breast cancers to find out whether determination of CDK1SA and CDK2SA could be useful for the prediction of patient outcomes. Although a high CDK1SA, a high CDK2SA and a high CDK2SA/CDK1SA ratio were significantly associated with a poor prognosis, the combination of these parameters (the CDK-based risk) has been found to predict patients' outcomes more accurately than each parameter alone. Multivariate analysis demonstrated that CDK-based risk was a significant prognostic indicator. More importantly, CDK-based risk was a highly significant and independent prognostic indicator for node-negative breast cancers.

The strength of this new indicator, CDK-based risk, is that it classified as many as 61% (109/178) of node-negative patients into the low-risk group where the RFS is extremely good, and the remaining 39% (69/178) into the high-risk group where the RFS is so low as to be equivalent to that seen in patients with one lymph node involvement [11]. This excellent capability for differentiation of the CDK-based risk sharply contrasts with that of St Gallen's risk classification of node-negative breast cancers. The latter categorized only 5% (8/166) of our subjects into the low-risk group, where recurrence was observed in 13% (1/8), and the remaining

95% (158/166) into the intermediate risk group, where recurrence was also observed in 13% (20/158).

We have focused on node-negative patients treated with hormonal therapy alone as systemic adjuvant therapy because this group represents the majority of node-negative cancers and includes some patients with unfavorable prognosis. For these patients, only the CDK-based risk was of significant use for the prediction of their prognosis (5yRFS 74.9% vs 98.4%). These findings seem to indicate that adjuvant hormonal therapy alone is under-treatment for node-negative and hormone receptor-positive patients with tumors belonging to the CDK-based high-risk group, who need chemotherapy in addition to hormonal therapy. By contrast, adjuvant hormonal therapy alone is an appropriate treatment for those in the CDK-based low-risk group. These preliminary findings obtained with a limited number of patients need to be confirmed in a future study including a larger number of patients.

Both CDK1 and CDK2 are considered to play an important role in cell proliferation and are expected to be associated with tumor aggressiveness and a poor prognosis [7, 8, 12, 13]. However, the prognostic impact of CDK1 in breast cancers still remains controversial [13–15]. Interestingly, some recent studies have shown that CDK1 may be required for apoptosis that is independent of the regulation of the cell cycle [16, 17]. Uncontrolled CDK1 activation might work as a brake for cancer cell growth in some tumors. Our present study has shown that a high ratio of CDK2SA to CDK1SA is associated with a poor prognosis and a low ratio is associated with a favorable prognosis. Although the real biological meaning of this ratio is still unclear, implication of CDK1 in apoptosis might partially explain why a low ratio of CDK2SA to CDK1SA is associated with a favorable prognosis. Several in vitro studies to clarify the biological meaning of this ratio are in progress in our laboratory.

Our results have demonstrated that tumors in the CDK-based high-risk group showed a significant association with unfavorable clinicopathologic features, such as high histologic grade, large tumor size, lymph node metastases and negative PR. CDK-based risk has a particularly strong association with histologic grade, suggesting that CDK-based risk may reflect the cell proliferation. It is well established that rapidly proliferating tumors are associated with a malignant potential to metastasize [4]. In fact, various parameters associated with cell growth have been identified as having the capability to serve as prognostic indicators in breast cancers. These parameters include mitotic index, DNA flow cytometry, ^{3}H-thymidine/5-bromo-2′-deoxyuridine uptake and Ki-67 antigen immunohistochemistry [18, 19]. The main problem inherent in these methods is that they are of a subjective nature with significant inter-observer or inter-assay variations, and are thus too difficult to standardize for use in routine laboratory tests. By contrast, determination of CDK1SA and CDK2SA can be accomplished with a well-standardized method ready for use in laboratory tests [9]. Another strength of CDK1SA and CDK2SA assay is that it needs only a very small sample (minimum 8 mm^{3}).

In conclusion, we have shown that CDK-based risk determined by evaluating CDK1SA and CDK2SA is strongly associated with clinical outcome especially for node-negative breast cancer patients. We consider that the CDK-based risk has potential as a new prognostic factor independent of the conventional risk factors, and as a routine laboratory test. However, our results need to be validated in a study with a larger number of patients on a multicenter basis.

references

1. Early Breast Cancer Trialists' Collaborative Group. Polychemotherapy for early breast cancer: an overview of the randomised trials. Lancet 1998; 352: 930–942.
2. Early Breast Cancer Trialists' Collaborative Group. Tamoxifen for early breast cancer: an overview of the randomised trials. Lancet 1998; 351: 1451–1467.
3. Clark GM. Do we really need prognostic factors for breast cancer? Breast Cancer Res Treat 1994; 30: 117–126.
4. Colozza M, Azambuja E, Cardoso F et al. Proliferative markers as prognostic and predictive tools in early breast cancer: where are we now? Ann Oncol 2005; 16: 1723–1739.
5. Keyomarsi K, Tucker SL, Buchholz TA et al. Cyclin E and survival in patients with breast cancer. N Engl J Med 2002; 347: 1566–1575.
6. Kuhling H, Alm P, Olsson H et al. Expression of cyclins E, A, and B, and prognosis in lymph node-negative breast cancer. J Pathol 2003; 199: 424–431.
7. Moroy T, Geisen C. Cyclin E. Int J Bioch Cell Biol 2004; 36: 1424–1439.
8. Sutherland RL, Musgrove EA. Cyclins and breast cancer. J Mammary Gland Biol Neoplasia 2004; 9: 95–104.
9. Ishihara H, Yoshida T, Kawasaki Y et al. A new cancer diagnostic system based on a CDK profiling technology. Biochim Biophys Acta 2005; 1741: 226–233.
10. Goldhirsch A, Glick JH, Gelber RD et al. Meeting highlights: international expert consensus on the primary therapy of early breast cancer 2005. Ann Oncol 2005; 16: 1569–1583.
11. Saez RA, McGuire WL, Clark GM. Prognostic factors in breast cancer. Semin Surg Oncol 1989; 5: 102–110.
12. Lee M-H, Yang H-Y. Regulators of G1 cyclin-dependent kinases and cancers. Cancer Metast Rev 2003; 22: 435–449.
13. Kourea HP, Koutras AK, Scopa CD et al. Expression of the cell cycle regulatory proteins p34^{cdc2}, p21^{waf1}, and p53 in node negative invasive ductal breast carcinoma. J Clin Pathol 2003; 56: 328–335.
14. Winters ZE, Hunt NC, Bradburn MJ et al. Subcellular localisation of cyclin B, Cdc2 and p21$^{WAF1/CIP1}$ in breast cancer: association with prognosis. Eur J Cancer 2001; 37: 2405–2412.
15. Umemura S, Komaki K, Noguchi S et al. Prognostic factors for node-negative breast cancers: results of a study program by the Japanese Breast Cancer Society. Breast Cancer 1998; 5: 243–249.
16. Castedo M, Perfettini J-L, Roumier T et al. Cyclin-dependent kinase-1: linking apoptosis to cell cycle and mitotic catastrophe. Cell Death Differ 2002; 9: 1287–1293.
17. Golsteyn RM. Cdk1 and Cdk2 complexes (cyclin dependent kinases) in apoptosis: a role beyond the cell cycle. Cancer Lett 2005; 217: 129–138.
18. Daidone MG, Silvestrini R. Prognostic and predictive role of proliferation indices in adjuvant therapy of breast cancer. J Natl Cancer Inst Monogr 2001; 30: 27–35.
19. Michels J-J, Marnay J, Delozier T et al. Proliferative activity in primary breast carcinomas is a salient prognostic factor. Cancer 2004; 100: 455–464.

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Original Article

Quantitative assessment of mammographic density and breast cancer risk for Japanese women

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Abstract

We conducted a case-control study to examine the relationship between breast density (BD) on mammography and breast cancer risk for postmenopausal Japanese women. The mammograms (205 cases and 223 controls) were classified by two doctors employing Wolfe's classification and used to measure BD with original computer software. A weak relationship between breast cancer risk and the parenchymal pattern of Wolfe's classification was found. The BD measured with the computer software, however, showed a significant relationship with breast cancer risk. Analysis after adjustment for epidemiologic factors showed that women in the quintile with the highest BD had a 3.02 times higher risk of breast cancer than those in the quintile with the lowest density. Since mammographic BD is clearly associated with breast cancer risk for postmenopausal Japanese women, our software can be expected to become a useful tool for objective risk assessment of breast cancer.

Keywords: Breast cancer; Breast density; Mammography; Breast cancer risk

Introduction

Correlation of breast cancer risk and breast pattern on mammograms was first reported by Wolfe in 1976, who classified mammograms into four categories according to their parenchymal patterns.[1] Since then, many other studies of mammographic patterns and breast cancer risk have been published, and most of them reported findings similar to those by Wolfe.[2–6] However, it has been suggested that qualitative assessment of mammography may involve interobserver variance.[2] To overcome this problem, quantitative methods of measuring breast density (BD) have come into use, and significant correlations between mammographic density and breast cancer risk have been reported.[6–9] However, most of these reports concern breast cancer risks for women in Europe and North America, with only a few reports dealing with Asian women including Japanese.[10–13] Since recently the incidence of breast cancer has been increasing in Japan too,[14] identification of women at high risk of breast cancer has become an important issue. However, mammographic screening for breast cancer has come into wide use only these past several years in Japan. It is therefore necessary to confirm that mammographic density can be a risk factor for breast cancer and to find the most suitable method for estimating BD of Japanese women, who have generally smaller breasts than their western counterparts.

In view of these considerations, we created a computer program to measure the area occupied by the mammary gland, which uses simple and objective adjustment of individual mammograms by means of comparison with the density of the pectoralis major muscle. With this program, we performed a case-control study to examine

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the relationship between BD and breast cancer risk for Japanese women.

Materials and methods

Case-control study

To examine the relationship between BD and breast cancer risk for Japanese women, we conducted a case-control study of 325 consecutive postmenopausal female subjects, who underwent surgery for pathologically confirmed primary breast cancer at Osaka University Hospital between January 1999 and December 2003. Menopausal status was reviewed with clinical records confirmed by interview before surgery. We restricted the subjects to postmenopausal women who had experienced no menstruation for more than one year. Women who had undergone hysterectomy and/or oophorectomy were excluded from the study, because we could not confirm that bilateral oophorectomy was completed in all of them. Women whose mammograms were not suitable for analysis because they did not include the pectoralis major muscle, and those who showed bilateral breast cancer were also excluded, as were those who had received hormone replacement therapy (HRT) because their number was very small. Eventually, 205 women aged 50 years and over were selected as study subjects.

In addition, 250 healthy postmenopausal women aged 50 years and over who had received mammographic breast cancer screening at least twice in the preceding 2 years at the Teikoku Hotel Clinic in Osaka City during the same period were enrolled as controls. Women who had undergone any breast or ovarian surgery were excluded. All the women stated on the consultation questionnaire that they had not received estrogen replacement therapy. The final number of controls was 223.

The medio-lateral-oblique (MLO) view of the mammograms was used for this study because this view was recommended for breast cancer screening in Japan. A preoperative mammogram of the breast contralateral to the operated one was used for the cases, while a mammogram of the right or left breast was chosen at random for the controls. Epidemiologic risk factors for breast cancer, such as age at menopause, family history of breast cancer among first- and second-degree relatives, history of parity and body mass index (BMI), were obtained from clinical records for review.

Qualitative evaluation of mammograms

All mammograms were evaluated and classified into four groups, N1, P1, P2 and DY, according to Wolfe's classification[1] by two doctors, who were certified for breast cancer screening with mammography by the Central Committee for Quality Control of Mammographic Screening of Japan. The mammograms were arranged randomly and reviewed without access to the information about any of the women. Several months after the review, all the mammograms were reevaluated by the same observer without reference to the first reading.

Quantitative assessment of mammographic BD

All mammograms were digitized with a scanner using an 8-bit gray scale with a resolution of 200 DPI, and the data were stored in a workstation. Although the BD of two women may be assessed with the naked eye as almost equal, the actual density in one can be higher than in the other when the intensity of part of the pectoralis major muscle in the former is lower than that in the latter. To overcome this problem, an original computer program to measure BD was newly created by T.S. and M.T. using the method shown in Fig. 1. First, the areas of the breast and pectoralis major muscle were manually identified on the monitor. A histogram showing pixel intensity (X-axis) and frequency (Y-axis) was created for each area. The pixel intensity was graded from 0 to 255, and the mean intensity (MI) of each area was obtained from the histogram. The average MI of the pectoralis major muscle (MIP) was calculated for a total of 60 mammograms, consisting of 30 mammograms each randomly selected from cases and controls. Since the average MI was 100, the area of the mammary gland on a representative mammogram with an MIP of 100 was determined by controlling the cutoff level of the intensity of the breast area for a comparison of the findings on the mammogram and on the monitor. The standard cutoff level of the intensity in the breast area was determined as 70, and the ratio of the area of the mammary gland to that of the entire breast was calculated as BD. However, cutoff levels differ among mammograms because of different conditions under which mammography is performed, so that we determined the cutoff level for calculation of BD according to the MIP of each individual mammogram. For example, when the MIP was 80, which is 20 less than the average MIP of 100, the cutoff level was set at 50, 20 less than the standard cutoff level of 70 (Fig. 1).

Clinicopathological features of breast cancer

For the cases, clinicopathological factors of breast cancer, such as tumor size, histological grade, HER2 overexpression, hormone receptor status and lymph node status, were obtained from clinical records for review.

Statistical analysis

The Mann–Whitney U test was used for statistical analysis to compare age distribution, and the student's T-test was used for a comparative analysis of the period after menopause and BMI of cases and controls. The Chi-square test was used for analysis of other epidemiologic factors. The relationships between parenchymal patterns and breast cancer risk, and between BD and breast cancer risk were analyzed with a logistic regression to obtain odds

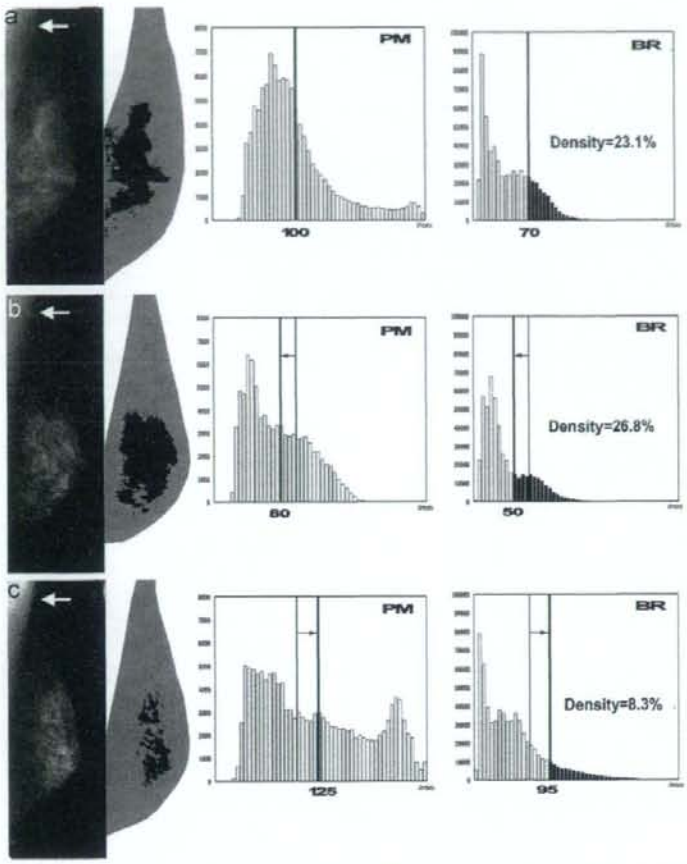

Fig. 1. The method for measurement of breast density. The areas of the breast (BR) and pectoralis major muscle (PM) were manually identified, and a histogram of pixel intensity (*X*-axis) and pixel frequency (*Y*-axis) was created for each area. In the standard case, the mean pixel intensity of the PM area was 100, and the standard cutoff level of the pixel intensity of the BR area was determined as 70 (a). In a case with the mean pixel intensity of the PM area at 80, the cutoff level was determined as 50 (b). In a case with the mean pixel intensity of the PM area at 125, the cutoff level was determined as 95 (c). The breast density calculated was 23.1% in the case a, 26.8% in b and 8.3% in c, while they were classified as P2 according to Wolfe's classification with the naked eye by the doctors.

ratios (OR) and 95% confidential intervals (CI). For the former analysis, the kappa statistic was used to determine interobserver and intraobserver agreement. For the latter analysis, controls were divided into five groups in order of BD, with almost equal numbers in each group. Next, the cases were divided into five groups according to BD corresponding to that of the five control groups. The relationship between BD and epidemiologic factors was then analyzed with the Mann–Whitney U test. Controls and cases were further divided into two groups each according to mean age, mean period after menopause and mean BMI of the study population, as well as the presence of childbirth and family history of breast cancer among first- and second-degree relatives. Subset analyses of breast cancer risk were performed for BD in groups stratified according to epidemiologic factors. The relationship between pathological factors and BD was analyzed with the Mann–Whitney U test. SPSS software (SPSS Japan Inc., Tokyo) was used for all statistical calculations.

Results

The background for epidemiologic risk factors of the study population is shown in Table 1. The mean age of the cases was 58.1 years, which was younger than that of controls (61.3 years; $P<0.01$), while the period after menopause was longer ($P<0.01$). There were no differences between the two groups as to BMI, history of parity and family history of breast cancer.

Table 1
Characteristics of study population.

	Cases ($N = 205$), number (%)	Controls ($N = 223$), number (%)	P
Age (mean ± SE, years)	50–93 (58.1 ± 5.2)	50–83 (61.3 ± 7.4)	<0.01
Period after menopause (mean ± SE, years)	1–52 (10.8 ± 0.57)	1–31 (8.8 ± 0.42)	<0.01
BMI (mean ± SE, kg m^2)	16.0–33.8 (22.9 ± 0.22)	16.6–35.2 (22.4 ± 0.18)	0.06
Parity			
None	28 (13.7)	37 (16.6)	0.40
> = 1	177 (86.3)	186 (83.4)	
Family history[a]			
No	170 (82.9)	192 (86.1)	0.21
Yes	35 (17.1)	28 (12.6)	
Unknown	0 (0)	3 (1.3)	

[a]Family history of breast cancer among first- and second-degree relatives.

Table 2
Relationship between qualitative evaluation using Wolfe's classification and breast cancer risk.

Wolfe's classification	Cases No. (%)	Controls No. (%)	OR [95% CI][a]	OR [95% CI][b]
Dr. A				
N1	33 (14.8)	28 (13.7)	1.00	1.00
P1	72 (32.3)	85 (41.5)	1.41 [0.76–2.61]	1.70 [0.89–3.24]
P2	110 (49.3)	79 (38.5)	1.03 [0.56–1.90]	1.31 [0.69–2.50]
DY	8 (3.6)	13 (6.3)	2.64 [0.93–7.49]	4.24 [1.51–15.3]
Dr. B				
N1	12 (5.4)	22 (10.7)	1.00	1.00
P1	85 (38.1)	76 (37.1)	0.49 [0.22–1.10]	0.55 [0.24–1.23]
P2	122 (54.7)	96 (46.8)	0.53 [0.24–1.17]	0.66 [0.29–1.49]
DY	4 (1.8)	11 (5.4)	2.24 [0.57–8.85]	2.74 [0.67–11.2]

[a]Odds ratio adjusted for age [95% confidential interval].
[b]Odds ratio adjusted for age, period after menopause, BMI, family history and parity [95% confidential interval].

Relationship between evaluation of mammography with Wolfe's classification and breast cancer risk

Table 2 shows the relationship between breast cancer risk and mammographic breast pattern determined by two doctors using Wolfe's classification. According to the grouping by doctor A, analysis after adjustment for age and epidemiologic factors as listed in Table 1 (OR = 4.24, 95% CI = 1.51–15.3) showed that women classified as DY had a higher risk of breast cancer than did those classified as N1. The groups classified by Dr. B, however, showed no significant relationship between breast patterns and breast cancer risk, while the OR of breast cancer risk for the DY group was more than twice that for the N1 group. The re-analysis based on the reevaluation of the mammograms performed several months after the first analysis by the same observers showed similar result (data not shown).

Interobserver and intraobserver agreement of breast patterns

The rates of agreement between the reviewers were 0.733 (kappa coefficient = 0.572) for the first review and 0.806 (kappa coefficient = 0.624) for the second. The rate of intraobserver agreement for Dr. A was 0.679 (kappa coefficient = 0.477) and 0.742 (kappa coefficient = 0.547) for Dr. B. These results indicate moderate inter- and intraobserver agreement.

Relationship between BD measured by computer and breast cancer risk

The results of the case-control study for breast cancer risk and BD measured with the computer software are summarized in Tables 3A and B. Women grouped in the quintile with the highest BD were found to have significantly higher risk of breast cancer than those in the quintile with the lowest density after adjustment for age and other epidemiologic factors (OR = 3.02, 95% CI = 1.58–5.77) (Table 3A). Comparison of the women in the quintile with the highest density (High) and those in all other quintiles (Low) yielded a risk ratio for breast cancer of 2.81 (95% CI = 1.72–4.59) for the "High" group after adjustment for age and other epidemiologic parameters (Table 3B).

Table 3
Relationship between breast density measured with computer and breast cancer risk comparing (A) each quintile to that with the lowest density (B) the quintile with the highest density to others.

Breast density (%)	Controls, no. (%)	Cases, no. (%)	OR [95% CI][a]	OR [95% CI][b]
(A)				
<3.4	45 (20.2)	42 (20.6)	1.00	1.00
3.4–8.8	44 (19.7)	32 (15.6)	0.89 [0.47–1.72]	0.98 [0.51–1.91]
8.9–16.5	45 (20.2)	30 (14.6)	0.84 [0.43–1.61]	0.94 [0.48–1.84]
16.6–28.7	45 (20.2)	37 (18.0)	1.04 [0.55–1.98]	1.36 [0.70–2.65]
28.8<	44 (19.7)	64 (31.2)	2.36 [1.28–4.37]	3.02 [1.58–5.77]
(B)				
Low (0–28.7)	179 (80.3)	141 (68.8)	1.00	1.00
High (28.7<)	44 (19.7)	64 (31.2)	2.51 [1.56–4.02]	2.81 [1.72–4.59]

[a]Odds ratio adjusted for age [95% confidential interval].
[b]Odds ratio adjusted for age, period after menopause, BMI, family history and parity [95% confidential interval].

Table 4
Relationship between epidemiologic factors and breast density measured with computer.

	Controls, % density (mean)	*P*	Cases, % density (mean)	*P*
Age (years)				
50–59	0.0–77.4 (13.8)	0.35	0.0–86.0 (26.8)	<0.01
> = 60	0.0–65.8 (11.6)		0.0–65.0 (11.8)	
Period after menopause (years)				
<9	0.0–77.4 (18.1)	0.09	0.0–76.7 (27.6)	<0.01
= >9	0.0–65.8 (14.7)		0.0–86.0 (17.9)	
BMI (kg/m^2)				
<22.3	0.0–77.4 (19.5)	<0.01	0.2–75.7 (26.6)	<0.01
= >22.3	0.0–56.8 (12.6)		0.0–86.0 (18.6)	
Parity				
None	0.0–77.4 (22.4)	<0.01	0.0–51.9 (23.2)	0.55
> = 1	0.0–65.8 (15.7)		0.0–86.0 (22.7)	
Family history				
No	0.4–77.4 (16.7)	0.63	0.0–86.0 (22.8)	0.80
Yes	0.0–65.8 (17.7)		0.0–64.7 (22.3)	

Relationship between BD and epidemiologic factors is shown in Table 4. BD was significantly higher in younger postmenopausal cases, while no age difference was observed in controls. BD was also higher in cases with a short period after menopause, in both cases and controls with low BMI, and in nulliparous controls.

Table 5 shows the relationship between breast cancer risk and BD when the subjects were stratified according to epidemiologic factors. This analysis showed that BD can be a risk factor of breast cancer for women from 50 to 59 years old who have become amenorrheic within the previous 9 years, whose BMI is 22.3 kg/m^2 or higher, who have given birth to at least one child and who have no family history of breast cancer among first- and second-degree relatives.

Relationship between BD measured with computer and clinicopathological factors of breast cancer

The relationship between BD and clinicopathological factors of breast cancer for the cases examined is shown in Table 6. No relationship was detected between BD and expression of hormone receptors, histological grade of the tumor or status of axillary lymph nodes, but BD was significantly higher in patients with HER2-positive than in those with HER2-negative tumors ($P<0.05$).

Discussion

Identification of women at high risk for breast cancer is important for efficient screening and effective prevention. Since Wolfe reported on the relationship between BD measured on mammograms and breast cancer risk for women,[1,5] many papers confirming this finding have been published.[2–4,6–10,15] In North America and Europe, mammographic screening for breast cancer is widely used, so that BD observed on mammograms is a suitable tool for assessment of breast cancer risk. In Japan, the use of mammography for public screening was started only recently at numerous clinics and hospitals in response to recommendations by the government because of an increase in the incidence of breast cancer.[14] The breasts

Table 5
Relationship between breast density measured with computer and breast cancer risk stratified with epidemiologic factors.

Age (years)	Density	Controls, no. (%)	Cases, no. (%)	OR [95% CI][a]	OR [95% CI][b]
(1) Age					
50–59	Low	116 (78.4)	52 (53.6)	1.00	1.00
	High	32 (21.6)	45 (46.4)	3.23 [1.84–5.69]	3.53 [1.96–6.35]
60–69	Low	55 (83.3)	69 (73.4)	1.00	1.00
	High	11 (16.7)	25 (26.6)	1.35 [0.56–3.29]	1.46 [0.61–3.47]
Period	Density	Controls, no. (%)	Cases, no. (%)	OR [95% CI][a]	OR [95% CI][c]
(2) Period after menopause					
<9	Low	107 (76.4)	60 (58.8)	1.00	1.00
	High	33 (23.6)	42 (41.2)	2.64 [1.48–4.70]	2.86 [1.58–5.19]
= >9	Low	72 (86.7)	81 (78.6)	1.00	1.00
	High	11 (13.3)	22 (21.4)	2.30 [0.99–5.28]	2.62 [1.11–6.18]
BMI	Density	Controls, no. (%)	Cases, no. (%)	OR [95% CI][a]	OR [95% CI][d]
(3) BMI					
<22.3	Low	105 (76.6)	66 (62.3)	1.00	1.00
	High	32 (23.4)	40 (37.7)	2.49 [1.38–4.49]	2.50 [1.38–4.54]
= >22.3	Low	74 (86.0)	75 (75.8)	1.00	1.00
	High	12 (14.0)	24 (24.2)	2.97 [1.31–6.31]	3.27 [1.40–7.62]
Parity	Density	Controls, no. (%)	Cases, no. (%)	OR [95% CI][a]	OR [95% CI][e]
(4) Parity					
Yes	Low	153 (82.3)	33 (38.4)	1.00	1.00
	High	33 (17.7)	53 (61.6)	2.72 [1.63–4.61]	2.90 [1.69–5.00]
No	Low	26 (70.3)	17 (60.7)	1.00	1.00
	High	11 (29.7)	11 (39.3)	1.97 [0.66–5.88]	2.28 [0.68–7.62]
Family history	Density	Controls, no. (%)	Cases, no. (%)	OR [95% CI][a]	OR [95% CI][b]
(5) Family history of breast cancer among first- and second-degree relatives					
Yes	Low	22 (78.6)	24 (68.6)	1.00	1.00
	High	6 (21.4)	11 (31.4)	2.26 [0.65–7.82]	2.24 [0.60–8.29]
No	Low	155 (80.7)	37 (41.1)	1.00	1.00
	High	37 (19.3)	53 (58.9)	2.58 [1.54–4.33]	2.88 [1.70–4.91]

[a]Odds ratio adjusted for age [95% confidential interval].
[b]Odds ratio adjusted for age, period after menopause, BMI and parity [95% confidential interval].
[c]Odds ratio adjusted for age, BMI, family history and parity [95% confidential interval].
[d]Odds ratio adjusted for age, period after menopause, family history and parity [95% confidential interval].
[e]Odds ratio adjusted for age, period after menopause, BMI and family history [95% confidential interval].

of most Japanese women are comparatively small, while the incidence of breast cancer in Japan is still lower than that in the Western countries.[16] It therefore seems to be interesting as well as timely to determine whether BD can be a risk factor of breast cancer for Japanese women, prompting us to conduct a case-control study for this purpose. To date, only a few reports have been published about the relationship between BD and breast cancer risk of Japanese women.[11–13] Nagao et al. reported on a matched case-control study evaluating mammographic density with both visual and computer-assisted methods. They reported that a group classified as DY based on Wolfe's classification showed a 2.2 fold higher risk of breast cancer compared to the N1 group, while P1 and P2 showed no such differences. These results were similar to ours, which were evaluated by two doctors using the same classification. The DY group showed a 2.74–4.24 fold higher risk of breast cancer compared to the N1 group. However, statistical significance was observed in the evaluation of only one doctor, while the P1 and P2 groups as evaluated by either doctor showed no significant difference in breast cancer risk. The kappa coefficient for the two doctors was 0.572, which indicates moderate agreement. This can be explained by the fact that assessment of mammographic pattern was difficult in some cases especially those with small breasts. Moreover, inter- and intraobserver variability may influence subjective classification.[2,17] A meta-analysis by McCormack et al. disclosed that relative risk of breast cancer in Caucasian women tended to increase with an increase in Wolfe's

Table 6
Relationship between breast density measured with computer and clinicopathological factors of breast cancer.

Clinicopathological factors	Number of patients	Breast density (%)	P-value
Estrogen receptor			0.90
Positive	123	22.3	
Negative	61	24.2	
Progesterone receptor			0.45
Positive	68	20.1	
Negative	116	24.6	
Tumor size			0.82
< = 2 cm	68	22.3	
>2 cm	98	21.6	
Histological grade			0.80
I	48	22.1	
II	87	22.1	
III	35	21.0	
Lymph node metastasis			0.39
Positive	54	24.6	
Negative	122	21.4	
HER2 overexpression			<0.05
Positive	15	32.8	
Negative	95	20.1	

grade, and was generally higher than that of Japanese women.[18] Carmen et al. reported that BD appears to be higher in Asian than in Caucasian women even when controlling for BMI and age.[19] Diversity of ethnicity associated with breast size and body fat may also affect the subjective assessment of mammographic patterns.

On the other hand, quantitative assessment of BD on a mammogram, which can evaluate the amount of fibroglandular tissue more objectively, is a promising method for breast cancer risk assessment. Boyd et al. used a method to trace the radiologically dense area,[8] and Byng et al. developed a technique based on an interactive thresholding method.[17,20] In addition, there have been several reports of original techniques for measuring BD on digitized mammograms.[21–23] However, radiographic conditions may affect selection of mammographically dense areas when an interactive method is used. When a breast contains extensive dense fibroglandular tissues, recently developed radiocameras can control the conditions automatically to show the internal structure of the dense tissue, so that the margin of the dense tissue, which is thinner than the central area, often becomes faint. In such cases, BD may be underestimated. Furthermore, most of these techniques use cranio-caudal views of mammograms, which often fail to show the whole breast area in women with small breasts, such as Japanese women.

For these reasons, we developed computer software for more objective assessment of mammogaraphic BD in terms of the ratio of the dense area to that of a whole breast. To this purpose, we used a MLO view, because it includes the pectoralis major muscle, which we used as a reference for adjusting the conditions of the mammograms. On the basis of the findings of a preliminary study, we used histograms for controlling the intensity of digitized mammograms by adjusting the MI of the pectoralis major muscle, and determined the threshold to select dense areas. This method is quite simple and objective, because the threshold is automatically calculated according to the MI of the pectoralis major muscle without any interaction by an observer. This density was found to represent a significant breast cancer risk factor for the women in the quintile with the highest BD (>28.7%) in the current study. The risk ratio was 3.02 compared to that for women with the lowest density (<3.4%) and 2.81 compared to women with a BD lower than 28.7% after adjustment for age and other epidemiologic factors. Nagata et al. reported that the risk ratio of breast cancer increased for breast densities of 25–50% (risk ratio = 3.0) and 50–100% (risk ratio = 4.2) in postmenopausal women.[13] They used their original computer software to divide a given breast area into four categories of density and calculate the density as a percentage.[24] The number of postmenopausal cases in their study was 75, which is considerably smaller than ours, although the controls comprised 289 women. These differences in conditions may account for the small differences between our and their results. In spite of the differences, BD measured objectively by computer software is generally considered a significant risk factor of breast cancer in Japanese women.

The BD measured with our method may theoretically not be accurate but only approximate because of individual differences in the thickness of the pectoralis major muscle. Furthermore, a whole breast area, which we selected for evaluation, contains retromammary fat tissue as well as the skin and subcutaneous fat tissue. Breast densities, that is, the ratios of the dense area to that of a whole breast, determined in our study became therefore smaller than those previously reported by other investigators.[13,25] Nevertheless, our simple method could identify Japanese women at high risk by using an MLO view for mammography, while the volume metric method may be useful for more accurate estimation of BD.[26]

As for factors influencing BD, BMI, history of parity and family history were significant for the controls, while age, period after menopause and BMI were significant for the cases in our study. Our subset analysis showed that BD can be a risk factor of breast cancer for women from 50–59 years old, who have experienced childbirth at least once and who have no family history of breast cancer among first- or second-degree relatives. Japanese women who have given birth to at least one child, and who have no family history are considered to be at lower risk for breast cancer than those who are nulliparous and have some family history of breast cancer.[27] Especially for such women, BD can thus be a useful tool for breast cancer risk assessment. On the other hand, BMI is considered to be a significant risk factor for breast cancer in postmenopausal women, that is, the higher the BMI, the higher the risk for breast cancer.[27,28] Interestingly, BD decreased with an increase in BMI in our study, as was also reported previously.[29,30]

Thus, a woman with high BMI whose BD remains high may run an additional risk for breast cancer. The relative risk of breast cancer for women with higher BMI tended to be higher than that for women with lower BMI in our study when adjusted for other epidemiologic factors.

Since BD may reflect the hormonal status of the mammary gland, it is of interest whether breast cancer related to high BD may have some special features in terms of receptors for estrogen and growth hormones. Several reports have dealt with this issue concerning Caucasian but not Japanese women. Ziv et al. reported that women with high BD had an increased risk of both estrogen-receptor positive and negative breast cancer.[31] Our data show the same relationship. On the other hand, Roubidoux et al. and Aiello et al. reported that tumor size significantly correlated with BD,[32,33] while our data do not show such a relationship. Porter et al. reported that tumor size did not vary significantly in cases of 'interval cancers', while screen-detected tumors were significantly smaller in fatty breasts.[34] The cases in our study included patients who found a lump by themselves and are considered 'interval cancers', and this may account for the difference. Other prognostic factors, such as lymph node status and histological grade, did not relate to BD as previously reported,[34] while BD was significantly higher in patients with HER2-positive cancer. HER2-positive breast cancer often occurs in young patients,[35,36] and our data showed that BD was higher in younger cases, so that this may be a cause of this finding. However, the number of samples was limited and the background of the cases varied widely. Further analysis of a greater number of patients is thus needed to determine the association of BD and clinico-pathological factors with hormonal features of the mammary gland such as serum and tissue estrogen levels.

In conclusion, our results show that BD is a significant risk factor of breast cancer for Japanese women, and that our method for measuring BD can be a useful tool for the selection of women with a high risk of breast cancer for intensive screening and some preventive programs. The technique can be easily used with a digital mammography system, which is recently becoming popular as a useful alternative to conventional screen/film mammography system. However, further studies of a larger population, multiethnic if possible, need to be performed to determine whether our method is universally applicable for breast cancer risk assessment.

Conflict of interest statement

None declared.

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References

1. Wolfe JN. Breast patterns as index of risk of breast cancer. *Am J Roentgenol* 1976;**126**:1130–9.
2. Boyd NF, O'Sullivan B, Campbell J, et al. Mammographic signs as risk factors for breast cancer. *Br J Cancer* 1982;**45**:185–93.
3. Brisson J, Merletti F, Sadowsky NL, Twaddle JA, Morrison AS, Cole P. Mammographic features of the breast and breast cancer risk. *Am J Epidemiol* 1982;**115**:428–37.
4. Brisson J, Morrison AS, Khalid N. Mammographic parenchymal features and breast cancer in the breast cancer detection demonstration project. *J Natl Cancer Inst* 1988;**80**:1534–40.
5. Wolfe JN, Saftlas AF, Salane M. Mammographic parenchymal patterns and quantitative evaluation of mammographic densities: a case-control study. *Am J Roentgenol* 1987;**148**:1087–92.
6. Byrne C, Schairer C, Wolfe J, et al. Mammographic features and breast cancer risk: effects with time, age, and menopause status. *J Natl Cancer Inst* 1995;**87**:1622–9.
7. Saftlas AF, Hoover RN, Brinton LA, et al. Mammographic densities and risk of breast cancer. *Cancer* 1991;**67**:2883–8.
8. Boyd NF, Byng JW, Jong RA, et al. Quantitative classification of mammographic densities and breast cancer risk: results from the Canadian national breast screening study. *J Natl Cancer Inst* 1995; **87**:670–5.
9. Kato I, Beinart C, Bleich A, Shaun S, Kim M, Toniolo PG. A nested case-control study of mammographic patterns, breast volume, and breast cancer (New York City, NY, United States). *Cancer Causes Control* 1995;**6**:431–8.
10. Maskarinec G, Meng L. A case-control study of mammographic densities in Hawaii. *Breast Cancer Res Treat* 2000;**63**:153–61.
11. Kojima O, Majima T, Uehara Y, et al. Radiographic parenchymal patterns in Japanese females as a risk factor for breast carcinoma. *World J Surg* 1984;**8**:414–8.
12. Nagao Y, Kawaguchi Y, Sugiyama Y, et al. Relationship between mammographic density and the risk of breast cancer in Japanese women: a case-control study. *Breast Cancer* 2003;**10**:228–33.
13. Nagata C, Matsubara H, Fujita H, et al. Mammographic density and the risk of breast cancer in Japanese women. *Br J Cancer* 2005; **92**:2102–6.
14. Marugame T, Kamo K, Katanoda K, Ajiki W, Sobue T. Cancer incidence and incidence rates in Japan in 2000: estimates based on data from 11 population-based cancer registries. *Jpn J Clin Oncol* 2006;**36**:668–75.
15. Tice JA, Cummings SR, Ziv E, Kerlikowske K. Mammographic breast density and the Gail model for breast cancer risk prediction in a screening population. *Breast Cancer Res Treat* 2005;**94**: 115–22.
16. Tamakoshi K, Yatsuya H, Wakai K, et al. Impact of menstrual and reproductive factors on breast cancer risk in Japan: results of the JACC study. *Cancer Sci* 2005;**96**:57–62.
17. Byng JW, Yaffe MJ, Lockwood GA, Little LE, Tritchler DL, Boyd NF. Automated analysis of mammographic densities and breast carcinoma risk. *Cancer* 1997;**80**:66–74.
18. McCormack VA, dos Santos Silva I. Breast density and parenchymal patterns as markers of breast cancer risk: a meta-analysis. *Cancer Epidemiol Bilomarkers Prev* 2006;**15**:1159–69.
19. Carmen MG, Halpern EF, Kopans DB, et al. Mammographic breast density and race. *Am J Roentgenol* 2007;**188**:1147–50.
20. Byng JW, Boyd NF, Fishell E, John RA, Yaffe MJ. The quantitative analysis of mammographic densities. *Phys Med Biol* 1994;**39**: 1629–38.
21. Sivaramakrishna R, Obuchowsky NA, Chilcote WA, Powell KA. Automatic segmentation of mammographic density. *Acad Radiol* 2001;**8**:250–6.
22. Chang YH, Wang XH, Hardesty LA, Chang TS. Computerized assessment of tissue composition on digitized mammograms. *Acad Radiol* 2002;**9**:899–905.

23. Jamal N, Ng KH, Looi LM, et al. Quantitative assessment of breast density from digitized mammograms into Tabar's patterns. *Phys Med Biol* 2006;**51**:5843–57.
24. Matsubara T, Yamazaki D, Fujita H, Hara T, Iwase T, Endo T. An automated classification method for mammograms based on evaluation of fibroglandular breast tissue density. In: Yaffe MJ, editor. *Proceedings of fifth international workshop on digital mammography*. Madison, WI: Medical Physics Publishing; 2000. p. 737–42.
25. Maskarinec G, Pagano I, Lurie G, Wilkens LR, Kolonel LN. Mammographic density and breast cancer risk. The multiethnic cohort study. *Am J Epidemiol* 2005;**162**:743–52.
26. Pawluczyk O, Augustine BJ, Yaffe MJ, Rico D, Yang J, Mawdsley E. A volumetric method for estimation of breast density on digitized screen-film mammograms. *Med Phys* 2003;**30**:352–64.
27. Kato I, Miura S, Kasumi F, et al. A case-control study of breast cancer Japanese women: with special reference to family history and reproductive and dietary factors. *Breast Cancer Res Treat* 1982;**24**:51–9.
28. Key TJ, Appleby PN, Reeves GK, et al. Body mass index, serum sex hormones, and breast cancer risk in postmenopausal women. *J Natl Cancer Inst* 2003;**95**:1218–26.
29. Titus-Ernstoff L, Tosteson ANA, Kasales C, et al. Breast cancer risk factors in relation to breast density (United States). *Cancer Causes Control* 2006;**17**:1281–90.
30. Boyd NF, Martin LJ, Sun L, et al. Body size, mammographic density, and breast cancer risk. *Cancer Epidemiol Biomarkers Prev* 2006;**15**:2086–92.
31. Ziv E, Tice J, Smith-Bindman R, Shepherd J, Cummings S. Mammographic density and estrogen receptor status of breast cancer. *Cancer Epidemiol Biomarkers Prev* 2004;**13**:2090–5.
32. Roubidoux MA, Bailey JE, Wray LA, Helvie MA. Invasive cancer detected after breast cancer screening yielded a negative result: relationship of mammographic density to tumor prognostic factors. *Radiology* 2004;**230**:42–8.
33. Aiello EJ, Buist DSM, White E, Porter PL. Association between mammographic breast density and breast cancer tumor characteristics. *Cancer Epidemiol Biomarkers Prev* 2005;**14**(3):662–8.
34. Porter GJR, Evans AJ, Cornford EJ, et al. Influence of mammographic parenchymal patterns in screening—detected and interval invasive breast cancers on pathologic features, mammographic features, and patient survival. *Am J Roentgenol* 2007;**188**:676–83.
35. Sjogren S, Inganas M, Lindgren A, Holmberg L, Bergh J. Prognostic and predictive value of c-erbB-2 overexpression in primary breast cancer, alone and combination with other prognostic markers. *J Clin Oncol* 1998;**16**:462–9.
36. Seo BK, Pisano ED, Kuzimak CM, et al. Correlation of HER-2/neu overexpression with mammography and age distribution in primary breast carcinomas. *Acad Radiol* 2006;**13**:1211–8.

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Topoisomerase IIalpha-positive and BRCA1-negative phenotype: Association with favorable response to epirubicin-based regimens for human breast cancers

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Abstract

Epirubicin exerts its anti-tumor effect through binding to topoisomerase IIalpha (TOP2A) and inducing DNA double-strand breaks. BRCA1 is involved in the repair of these breaks. We investigated the relationship between TOP2A or BRCA1 immunohistochemical expression and pathological response in 108 primary breast cancers treated with epirubicin-based regimens. The pCR (pathological complete response) rate for TOP2A-positive (17%) was significantly ($P < 0.005$) higher than for TOP2A-negative (2%), while the pCR rate for BRCA1-negative (11%) was non-significantly higher than for BRCA1-positive (5%). The pCR rate of TOP2A-positive and BRCA1-negative (30%) was significantly higher than for TOP2A-negative and BRCA1-positive (3%; $P < 0.05$), or TOP2A-negative and BRCA1-negative (0%; $P < 0.005$). The TOP2A-positive and BRCA1-negative phenotype associates with a favorable response to epirubicin-based regimens.

Keywords: BRCA1; Breast cancer; Epirubicin; Pathological response; Topoisomerase IIalpha

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1. Introduction

Epirubicin, which belongs to the anthracycline family, is one of the most aggressive drugs against breast cancer and epirubicin-based regimens such as 5-FU plus epirubicin pulse cyclophosphamide (FEC) and epirubicin plus cyclophosphamide (EC) are widely used in adjuvant and neoadjuvant as well as in metastatic settings. These epirubicin-based regimes, however, although very active, are not necessarily effective for all patients. In fact, response rates of metastatic breast cancers to epirubucin-based regimens reportedly range from 50% to 60% [1,2]. On the other hand, adverse events such as leucopenia and alopecia are observed in virtually all patients treated with these regimens although their severity differs from patient to patient. In addition, a small but significant proportion of patients develop serious adverse events such as cardiac failure and myeloproliferative diseases. In order to increase the efficiency of chemotherapy and avoid unnecessary adverse events, it is therefore very important to administer chemotherapy to those patients who are likely to respond and not to those who are unlikely to respond. For purpose, reliable predictive factors for response to chemotherapy need to be developed. Until now, various biological parameters, including HER-2 [3], p-glycoprotein [4], p53 [5], estrogen receptor (ER) [6], S-phase fraction [7], Ki-67 [7], have been proposed as candidate predictive factors for response to epirubicin-based and doxorubicin-based regimens (doxorubicin is another anthracycline) but their clinical value remains controversial so that they have not yet been integrated in daily practice.

Among the predictive factors so far studied, *HER-2* gene amplification and HER-2 overexpression have been attracting a great deal of attention, and a significant association between *HER-2* gene amplification or HER-2 overexpression and a favorable response to epirubicin-based regimens has been reported [3,8,9]. However, recent studies have shown that such an association between response and HER-2 is indirect and that the direct association occurs between response and the expression of topoisomerase IIalpha (TOP2A), which is a target molecule of epirubicin [10,11]. The *TOP2A* gene is localized close to the *HER-2* gene and is often coamplified with the *HER-2* gene [12]. TOP2A plays a pivotal role in DNA replication and catalyzes the transport of one DNA double helix through another by the transient introduction of DNA double-strand breaks [13]. Anthracyclines including epirubicin and doxorubicin bind to TOP2A and stabilize the DNA double-strand breaks, resulting in cell cycle arrest and apoptosis [13,14]. In fact, an in vitro study has shown that breast cancer cells with TOP2A overexpression are more sensitive to doxorubicin [12]. It has also been reported that TOP2A expression is observed in 20–62% [11,15–19] and TOP2A gene amplification in 12–24% of human breast cancers [11,16,18,20,21]. Several lines of evidence have suggested that anti-tumor activity of the epirubicin-based regimens is associated with TOP2A expression or *TOP2A* gene amplification, although the contradictory results have also been reported [21–24].

In addition to TOP2A, BRCA1 has recently been gaining attention as a predictive factor for response to epirubicin-based regimens. BRCA1 plays an important role in double-strand DNA repair [25], and because epirubicin induces DNA double-strand breaks, it is possible that BRCA1 may modulate the response to epirubicin. In this connection, it has been reported that a mouse cell line deficient in BRCA1 displayed an increased sensitivity to the agents, including doxorubicin, which cause double-strand DNA breaks, and that induction of wild-type BRCA1 resulted in a reduced level of apoptotic cell death after treatment with DNA-damaging agents [26]. It has also been found that overexpression of BRCA1 in murine ovarian cancer cells increased the resistance to doxorubicin [27]. Furthermore, Delaloge et al. reported that 53% of locally advanced breast cancers carrying a BRCA1 mutation showed complete response to the anthracycline-based regimens while only 14% of sporadic breast cancers did, indicating that breast cancers lacking a BRCA1 function due to its mutation are more sensitive to anthracycline-based regimens [28]. Although BRCA1 mutation is rare, a significant proportion of sporadic breast cancers lack BRCA1 expression due to hypermethylation of the promoter region of the *BRCA1* gene [29], overexpression of HMGA1 [30], or overexpression of ID4 [31]. Thus, it is possible that BRCA1 expression may influence the sensitivity of sporadic breast cancers to epirubicin-based regimens. However, this possibility has hardly been investigated.

As mentioned earlier, it has been speculated that TOP2A and BRCA1 may be associated with sensitivity to epirubicin-based regimens, and thus are potentially useful as predictive factors for these regimens. Nevertheless, the association between

BRCA1 and response to epirubicin-based regimens in sporadic breast cancers has yet to be reported. This prompted us to immunohistochemically investigate TOP2A and BRCA1 expression simultaneously in breast cancer tissues obtained before the administration of epirubicin-based regimens (preoperative setting), and to study the relationship between the expression of these two markers and pathological response.

2. Materials and methods

2.1. Patients and tumor samples

For this study, 108 primary breast cancer patients at stage II ($n = 73$), III ($n = 22$), and IV ($n = 13$) were consecutively recruited. They were treated with epribucin-based regimens in the preoperative setting during the period between September 1999 and April 2004 at Osaka University Hospital, Osaka Medical Center for Cancer and Cardiovascular Diseases, and Kyushu University Hospital. Treatment with EC was used for 97 and with FEC for 11 patients and all of them were subsequently treated with breast conserving surgery or mastectomy. The epirubicin-based regimens were administered every 3 weeks for 3–6 cycles (3 cycles for 47 patients, 4 cycles for 45 patients, 5 cycles for one patient, and 6 cycles for seven patients). The remaining eight patients were treated with only 2 cycles of EC ($n = 5$) or FEC ($n = 3$) because of disease progression, and were switched to other chemotherapy (paclitaxel or docetaxel) before surgery. The dose of epirubicin for both the EC and FEC regimens was 60 mg/m^2 epirubicin for 107 patients and 100 mg/m^2 for one patient. Tumor tissue samples were obtained from primary tumors by means of vacuum-assisted core needle biopsy prior to preoperative chemotherapy. The samples were subjected to pathological diagnosis for determination of ER, PR, and HER-2 status as well as immunohistochemical study of TOP2A and BRCA1. This study was approved by the IRB of Osaka University Graduate School of Medicine.

2.2. Assessment of tumor grade and pathological response

Nuclear grade, mitotic score, and tubular formation were determined according to the criteria specified by Elston and Ellis [32]. Since the association between pathological response and patient prognosis is much stronger than that between clinical response and patient prognosis [33–35], we adopted pathological response, but not clinical response, to evaluate the effect of epirubicin-based regimens in the present study. Pathological response of breast tumors was evaluated in 100 patients who were treated with three or more cycles of the epirubicin-based regimens alone. Multiple slides prepared from primary breast tumors after preoperative chemotherapy were examined and chemotherapeutic effect was determined as for the breast tumors according to the criteria specified in the General Rules for Clinical and Pathological Recording of Breast Cancer 2005 [36]. These criteria define Grade 0 as no response (almost no change in cancer cells), Grade 1 as slight response (1a: mild changes in cancer cells regardless of the area; 1b: marked changes in one-third or more but less than two-thirds of tumor cells), Grade 2 as marked response (marked changes in two-thirds or more of tumor cells), and Grade 3 as complete response (necrosis or disappearance of all tumor cells). The eight patients who showed a progressive disease after 2 cycles of the epirubicin-based regimens and were switched to other types of chemotherapy were classified as pathological non-responders.

2.3. Immunohistochemistry of HER-2, TOP2A, and BRCA1 expression

The expression of HER-2, TOP2A, and BRCA1 was evaluated immunohistochemically by using the tumor specimens obtained as described under patients and tumor samples. Sections prepared from the formalin-fixed paraffin-embedded tumor specimens were deparaffinised and rehydrated in graded alcohol. Antigens were retrieved by incubating the sections in 10 mmol/l citrate buffer (pH 6.0) at 95 °C for 50 min for TOP2A or by boiling for 15 min in a microwave oven for BRCA1. After quenching endogenous peroxidase with 3% H_2O_2 in methanol for 20 min, the resultant slides were treated with Block Ace (Dainippon Sumitomo Pharmaceutical, Osaka, Japan) for 30 min at room temperature. The samples were then incubated overnight at 4 °C with a polyclonal rabbit anti-c-erbB2 antibody (1:100 dilution; Nichirei Biosciences Inc., Tokyo, Japan) for HER-2, with a mouse monoclonal anti-TOPOIIα antibody (1:70 dilution; KiS1, DakoCytomation Inc., Carpinteria, CA) for TOP2A, or with a mouse monoclonal anti-BRCA1 antibody (1:70 dilution; Ab-1, Oncogene Science, Cambridge, MA) for BRCA1. They were subsequently incubated at room temperature for 30 min with the ABC Kit (Vector Laboratories, Burlingame, CA) using biotinylated anti-rabbit immunoglobulin G antibody for HER-2 or biotinylated anti-mouse immunoglobulin G (IgG) antibody for BRCA1. For TOP2A, incubation was performed with EnVision+ System Peroxidase (DakoCytomation) according to the manufacturer's instructions. Finally, the antibody complex was visualized with 3,3′-diaminobenzidine tetrahydrochloride (Merck, Darmstadt, Germany) and the sections were counter-stained with hematoxylin.

Positive reactions for HER-2 were scored as four grades, as previously reported [37], according to the intensity and pattern of the staining. The four grades were: 0

(no or less than 10% membrane staining in tumor cells); 1+ (faint membrane staining in more than 10% of tumor cells, partial staining of the membrane); 2+ (weak-to-moderate but complete membrane staining in more than 10% of tumor cells); 3+ (strong and complete membrane staining in more than 10% of tumor cells) Grade 2+ and 3+ tumors were considered to be HER-2 positive. The most actively stained lesions were selected microscopically and nuclear staining was counted in 1000 cancer cells without knowledge of patients outcome, and 5% and 10% were used as the respective cut-off values for TOP2A and BRCA1 according to the method described previously [17,38].

2.4. ER and PR assay

ER and progesterone receptor (PR) protein levels in the tumor specimens obtained before preoperative chemotherapy were determined in 83 cases with immunohistochemistry (cut-off value was 10% for both ER and PR) or in 21 cases with an enzyme immunoassay using kit from Abbott Research Laboratories (Chicago, IL) according to the manufacturer's instructions (cut-off values for ER and PR were 13 and 10 fmol/mg, respectively).

2.5. Statistical methods

The relationship between clinicopathological or biological parameters and pathological response was evaluated with the Fisher's exact test. Multivariate analysis of the relationship of TOP2A and BRCA1 expression with pCR was determined using a logistic regression method to obtain the odds ratio and 95% confidence interval, being adjusted for menopausal status, tumor size, lymph node metastasis, distant metastasis, nuclear grade, ER, PR, and HER-2 status. Statistical significance was assumed for $P < 0.05$.

3. Results

3.1. Relationship between clinicopathological or biological parameters and pathological response to epirubicin-based regimens

Pathological response was divided into two categories, i.e., pathological complete response (pCR, Grade 3) and non-pCR (Grades 0, 1a, 1b, and 2) for evaluation of its relationship with clinicopathological parameters (Table 1). The pCR rate (13%) of small tumors (≤5 cm) was significantly ($P < 0.05$) higher than that (0%) of large tumors (>5 cm). No statistically significant association was observed between pCR rate and menopausal status, lymph node status, distant disease status, nuclear grade, mitotic score, or tubular formation.

Table 1
Relationship between clinicopathological factors and pathological response to epirubicin-based regimens

Pathological response[a]	Non-pCR	pCR	*P*-value
Menopausal status			
Pre-	62 (94)[b]	4 (6)	0.30
Post-	37 (88)	5 (12)	
Tumor size			
≤5 cm	58 (87)	9 (13)	<0.05
>5 cm	41 (100)	0 (0)	
Lymph node metastasis			
Negative	31 (91)	3 (9)	0.99
Positive	68 (92)	6 (8)	
Distant metastases			
Negative	86 (91)	9 (9)	0.59
Positive	13 (100)	0 (0)	
Nuclear grade			
I + II	45 (94)	3 (6)	0.71
III	45 (90)	5 (10)	
Unknown	9 (90)	1 (10)	
Mitotic score			
I + II	56 (95)	3 (5)	0.25
III	34 (87)	5 (13)	
Unknown	9 (90)	1 (10)	
Tubular formation			
I + II	19 (90)	2 (10)	0.67
III	71 (92)	6 (8)	
Unknown	9 (90)	1 (10)	

[a] Pathological response was classified as described in Section 2.
[b] % of patients.

The pathological response was further studied in terms of its relationship with biological parameters including ER, PR, and HER-2, but no significant association with any of these parameters was detected (Table 2).

Table 2
Relationship between biological parameters and pathological response to epirubicin-based regimens

Pathological response[a]	Non-pCR	pCR	*P*-value
Estrogen receptor			
Positive	28 (90)[b]	3 (10)	0.99
Negative	67 (92)	6 (8)	
Unknown	4 (100)	0 (0)	
Progesterone receptor			
Positive	28 (90)	3 (10)	0.99
Negative	51 (89)	6 (11)	
Unknown	20 (100)	0 (0)	
HER-2 status			
Positive	24 (89)	3 (11)	0.43
Negative	70 (93)	5 (7)	
Unknown	5 (83)	1 (17)	

[a] Pathological response was classified as described in Section 2.
[b] % of patients.

3.2. TOP2A and BRCA1 expression and their relationship with clinicopathological and biological parameters or pathological response

Expression of TOP2A and BRCA1 was examined immunohistochemically in 108 tumor samples obtained before preoperative chemotherapy. Representative immunohistochemical results are shown in Fig. 1. Tumors with a high mitotic score (III) were significantly more likely to show a higher TOP2A positivity than tumors with a low mitotic score (I + II) (67% vs. 29%, $P < 0.001$). Tumors with positive HER-2 were significantly more likely to show a higher TOP2A positivity than those with negative HER-2 (59% vs. 35%, $P < 0.05$) (Table 3). Tumors with

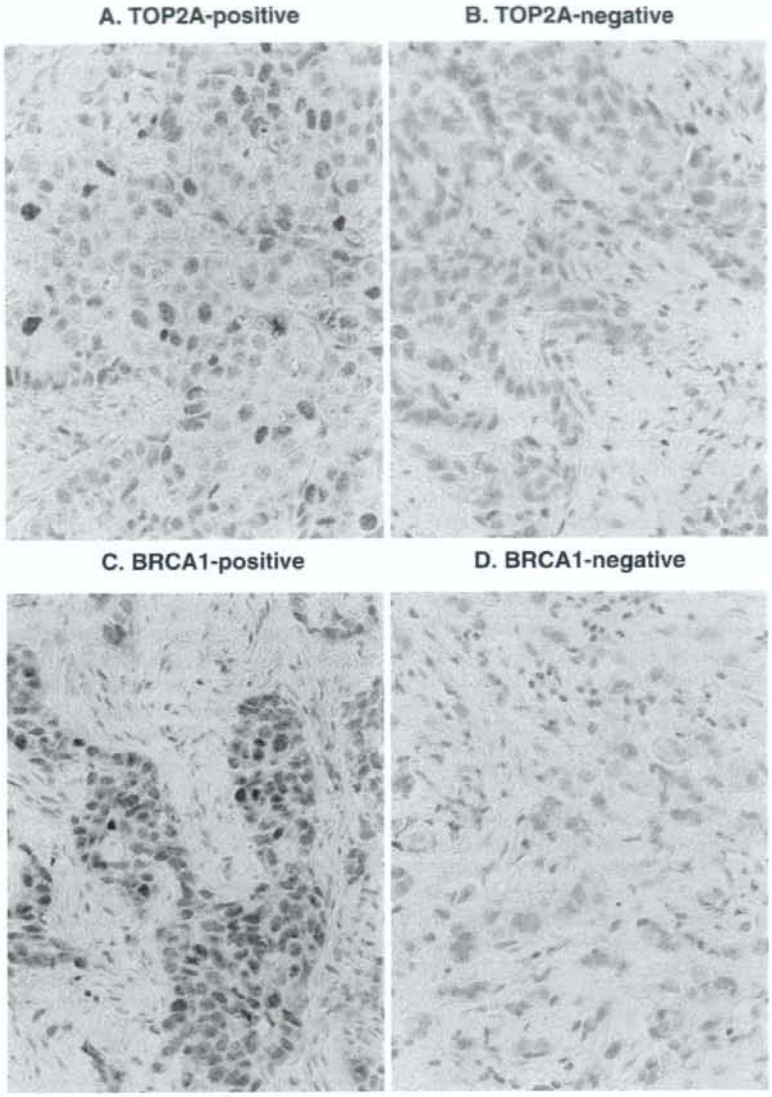

Fig. 1. Immunohistochemical staining of TOP2A and BRCA1. Representative results of immunohistochemical staining of TOP2A and BRCA1 (400×). Nuclear staining of TOP2A-positive (A), TOP2A-negative (B), BRCA1-positive (C), and BRCA1-negative (D) is seen in tumor cells.