Table 2. Pearson's correlations between clinicopathological features and androgen receptor (AR), 5α-reductase type 1 (5αR1), and 17β-hydroxysteroid dehydrogenase type 5 (17βHSD5) expression in combined cohort of patients with triple negative breast cancer from Japan and Thailand (n = 203)

	5αR1 score	17βHSD5 score	Age	Ki-67	Tumor diameter	Lymph node metastasis	Presence of distant metastasis
AR H score	***0.2151 (0.0020)***	***0.2438 (0.0010)***	***0.2948 (<0.0001)***	***−0.1414 (0.0480)***	**−0.1390 (0.0550)**	0.0920 (0.2040)	−0.0190 (0.7890)
5αR1 score		***0.4062 (<0.0010)***	0.0511 (0.4470)	***−0.2037 (0.0040)***	0.0405 (0.5780)	0.0343 (0.6380)	−0.0845 (0.2420)
17βHSD5 score			0.0740 (0.3010)	−0.0546 (0.4460)	0.0210 (0.7730)	−0.0292 (0.6870)	−0.0606 (0.4000)
Age				−0.0665 (0.3530)	−0.1274 (0.0780)	0.0319 (0.6600)	−0.1104 (0.1230)
Ki-67					−0.0205 (0.7780)	−0.0272 (0.7080)	0.0005 (0.9950)
Tumor diameter						***0.2808 (<0.0001)***	***0.1492 (0.0400)***
Lymph node invasion							**0.1395 (0.0540)**

Correlation strength was calculated using Pearson's Rho; significance (P-values) shown in parentheses. Bold, near significant correlation; bold italics, significant correlation.

separately, but only the Japanese cohort showed significance (P = 0.002) in separate analysis.

Analysis of the effects of AR and enzyme expression on DFS and OS. Interactions between AR, enzyme expression, and clinicopathological factors were modelled using a multivariate Cox proportional hazards model (Table 3). For OS, a total of 176 patients had complete data with 31 events; for DFS, a total of 174 patients were available with complete data with 35 events. For linear factors the risk ratio represents the change in risk per unit of the regressor, for ordinal factors the risk ratio is standardized to the lowest (i.e., in the least developed stage) grouping. In the stratification by country, the risk ratios were standardized to the Japanese cohort. Both models were significant predictors of outcome (OS, $P < 0.0001$; DFS, $P = 0.0005$). This analysis showed that the only robust and significant factors were tumor TNM stage at the time of diagnosis (which accounts for tumor diameter, lymph node involvement, and presence of distant metastasis), and Ki-67 LI ($P < 0.001$ and $P = 0.017$ respectively).

Results of univariate Kaplan–Meier analysis showed no significant effects on OS or DFS in evaluating AR+5αR1+ groups compared to others, but a significantly worse ($P < 0.05$) survival outcome was detected in AR-5αR1− patients in an 80-month follow-up period, which corresponds to the longest length of follow-up for patients in the Thai cohort (Fig. 3). The Ki-67 LI also predicted survival in the TNBC group in this analysis (Fig. 3).

Discussion

This study was undertaken to address controversies regarding the possible roles of androgenic pathways in the biological behavior of TNBC patients. In addition, we also hypothesized that assessing enzyme status in combination with receptor status could illuminate why contradictory results existed regarding the roles of androgen signalling in TNBC because of the biological importance of the intratumoral production of active steroids *in situ* (intracrinology).[62] In particular, we have previously shown that only the combination of the enzyme and AR expression (i.e., an intact androgen synthesis and signalling pathway) in tumor cells could predict a better clinical outcome for AR+ breast cancer patients, whereas AR status in tumor cells alone

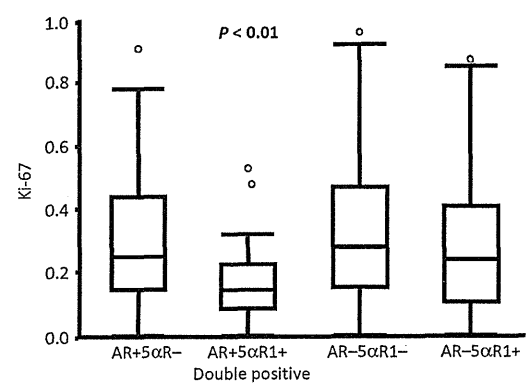

Fig. 2. Correlation between androgen receptor (AR) H score and Ki-67 labeling index (LI) and effects on Ki-67 LI when triple negative breast cancer cases were subdivided according to receptor and enzyme status. The AR+ 5α-reductase type 1 (5αR1)+ group was significantly different from the AR−5αR1− group (P = 0.004, Tukey–Kramer HSD), based on the cut-off points >50% immunopositivity in 5αR1 and >10% labeling index for AR. If these results were stratified by country of origin, only the Japanese cohorts showed statistically significant differences. Thai cohorts showed a similar trend but the correlation did not reach statistical significance. The circles above the columns represent outlying points.

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Table 3. Interactions between androgenic pathways and clinical factors in determining survival outcomes in triple negative breast cancer patients from Thailand and Japan (n = 203)

	Overall survival				Disease-free survival			
	P-value	Risk ratio	CI lower	CI upper	*P-value*	Risk ratio	CI lower	CI upper
Ki-67	**0.0175**	6.80	1.42	40.75	**0.0404**	4.798	1.07	22.45
TNM stage								
IIa	**<0.0001**	0.81	0.24	2.86	**<0.0001**	1.18	0.42	3.59
IIb		5.17	1.37	20.39		3.72	0.99	13.42
IIIa		5.86	1.30	25.32		5.19	1.42	18.33
IIIb		3.61	0.17	26.59		3.95	0.55	19.15
IIIc		6.95	1.98	25.48		6.99	2.08	23.64
IV		401.97	34.79	4646.53		98.81	11.40	658.97
Country	0.8429	0.88	0.23	3.03	0.3792	0.64	0.23	1.72
AR H score	0.4268	1.00	0.99	1.01	0.7099	1.00	0.99	1.01
5αR1 score								
1	0.5886	1.54	0.48	4.89	0.6304	1.47	0.52	4.17
2		0.93	0.31	2.88		0.97	0.35	2.66
17βHSD5 score								
1	0.8235	0.75	0.29	1.99	0.6275	0.67	0.29	1.58
2		0.97	0.29	3.04		0.93	0.29	2.67
Age	0.4145	2.89	0.98	1.05	0.7953	1.00	0.97	1.04

Bold indicates significant value. 5αR1, 5α-reductase type 1; 17βHSD5, 17β-hydroxysteroid dehydrogenase type 5; AR, androgen receptor; CI, confidence interval.

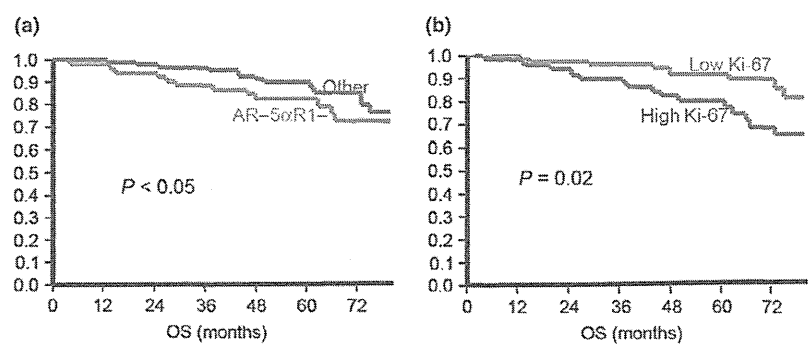

Fig. 3. Survival curves according to Ki-67 and androgen receptor (AR)−/5α-reductase type 1 (5αR1)− status of breast cancer patients. Kaplan–Meier survival curves analyzed according to 5αR1-/AR- (a) and Ki-67 labeling index (LI) (b). Double negative cases were tentatively defined as having less than 10% LI for AR and less than 50% of carcinoma areas immunohistochemically positive for 5αR1. Low Ki-67 cases were tentatively defined as less than 25% Ki-67 LI, with high Ki-67 cases greater than 25% LI (median value). Survival curves and analysis were truncated at 80 months because this was the longest time to follow up in the Thai cohort. If not truncated the survival curve of androgen action negative groups the patients crossed at approximately 90 months, but the Ki-67 survival curves did not cross until 150 months (the longest time of clinical follow-up in this study).

failed to indicate any significant effect of androgen signalling on prognosis of breast cancer patients.[59]

In our present study, all associations were tested by being stratified according to each cohort examined, as well as combined because of the differences in a number of clinicopathological factors between these two cohorts. This study was not designed to answer any possible cause of differences between the two cohorts, however, there are many factors we can speculate may be the cause, including but not limited to difference in ethnicity, and differences in methodological approaches between different hospitals. Despite these differences, the same trend was evident in terms of the possible correlation between various clinicopathological variables and those factors as related to androgenic signalling in tumor cells. Therefore, the underlying differences between these two cohorts are reasonably postulated not to hamper the validity of the conclusions in our study either when separated or combined.

Androgen receptor nuclear immunoreactivity was detected in approximately 25% of all TNBC cases examined, which fell into the ranges previously reported for TNBC.[45] In addition, AR immunoreactivity was associated with enzyme status, which is consistent with our previous findings in the whole series of breast cancer not stratified by individual subtypes.[59] The status of both AR and enzymes was independently associated with lower rates of Ki-67 LI of tumor cells and AR tended to be associated with a smaller tumor diameter, although the tendency did not reach statistical significance. These findings were also consistent with results of previously published studies regarding the correlation between AR and reduced Ki-67 LI in TNBC,[44,56] ER−,[23] and general breast cancer groups,[20] as well as the correlation between growth suppression in response to androgen therapy *in vivo*.[63,64] In addition, results of our present study indicated the presence of androgen synthesis enzymes in the tumors, which conferred an

additional contribution to decreased cell proliferation. It is also interesting to note that the lowest Ki-67 LI was indeed associated with AR+/5αR1+ cases followed by AR−/5aR1+, AR+/5aR1−, and AR−/5aR1− (Table 2). The statistically significant correlation between Ki-67 LI and survival was previously reported in TNBC cohorts,[65] which also suggests that AR and AR/enzyme expression may confer a survival advantage through the suppression of cell proliferation in TNBC.

In this study, it is also true that we could not show a significant effect of AR or enzyme expression on the overall clinical outcome of the patients, although the AR−5αR1− groups conferred an aggressive clinical course upon the patients examined in this study. One explanation for this finding could be that AR and androgen metabolism may not be the only factors responsible for cell proliferation, as in luminal A type breast cancer, but merely two of many governing cell proliferation. Hence, they may not necessarily be sufficient to significantly affect survival in our cohort, with its limited numbers of patients, especially in the AR+/enzyme+ group. The lack of correlation between AR and survival is not contradictory to published reports as, despite many studies showing a survival advantage associated with AR expression,[11,46,47] others have been unable to find a significant effect of AR expression on survival outcomes for patients.[20,43,48]

Results of the correlation analysis revealed that, aside from Ki-67, the only other clinical factor showing a statistically significant correlation with AR was patient age. This correlation has been detected in previous studies examining AR expression in breast cancer non- stratified by subtype,[7] ER− breast cancer,[46] and in TNBC,[56] although these results are not necessarily consistent.[19] A positive association between an increased level of circulating androgens in relation to estrogens and AR expression in the breast has been also reported in female to male transsexuals[66] and in prepubescent and postmenopausal primate breast tissue,[67] which suggest that correlations between age and AR expression may be explained by changes in the availability of circulating sex steroids. Further studies are needed to investigate what effect, if any, this increase in AR receptor expression with age may have on the underlying biology of TNBC. The lack of correlation between AR or enzyme expression and distant metastasis status or lymph node metastasis suggest that androgen signalling in TNBC cells might not play important roles in the process of tumor cell invasion and/or metastasis either to the lymphatic system or distant from the original tumor.

One of the major inconsistencies currently present in TNBC patients is the discrepancy between the majority of IHC results in TNBC cases and the results reported in AR+ TNBC cell lines. Although many clinical cohorts[11,46,47] and transient transfection[63] studies have shown clinically beneficial effects of AR expression in tumor cells, the great majority of AR+ TNBC cell lines, with the exception of MFM223 cells,[64] show growth stimulation or proliferation responses to androgen treatment,[57,68] mediated through activation of ER target genes[54] via androgen receptor actions.[48,52–55] Results of these *in vivo* studies suggest that AR expression in triple negative disease would be detrimental for the patients. The results are also in agreement with gene expression studies selecting for androgen-overexpressing ER− tumors, which indicated adverse clinical outcomes in OS[69] and disease recurrence.[56] Clinically these inconsistencies have posed serious problems in terms of deciding whether androgen inhibition or stimulation would prove beneficial in TNBC patients. Some potential explanations for the contradictions could be as follows.

First, there are often-raised differences between results in cell lines and human tissue specimens, as in other malignancies.[70–72] The three most widely used AR+ TNBC cell lines have significant mutations associated with intracellular signalling that are not necessarily recapitulated in the majority of TNBC specimens.[56,70,73,74] In addition, the most widely characterized TNBC cell line, MBD-MB-453, carries recently discovered mutations in the AR that alter its promiscuity to other ligands.[75] At this point the frequency of the mutation in the TNBC cancer population is totally unknown.

A second possible explanation is that the effects of AR expression in TNBC may not be homogeneous, or that gene expression profiling and IHC data of individual cases may not be selecting the same populations of patients. Gene expression profiling was initially used to show that breast cancer can be subtyped into categories that have meaningful clinical outcomes based upon their gene expression profiles.[76,77] Subsequent studies using gene expression profiles found groupings within breast cancer that are defined by the expression of AR in the absence of ER. These groupings have been termed molecular apocrine and LAR.[54,56] The molecular apocrine subtype is defined by the lack of a complete luminal or basal gene signature (as defined by Perou)[78] in combination with increment in AR signalling, whereas the LAR subtype is defined by enrichment of hormonally regulated pathways. It should be noted that the molecular apocrine classification included both HER2/ERBB2 enriched cancers in addition to triple negative cancers, and the triple negativity in the complete LAR set was defined by gene expression levels rather than a direct assessment of IHC reactivity of ER/PR and HER2. In both analyses, although the gene expression profiles selected for subgroups contained high levels of AR,[56,69] not all AR-expressing tumors were included in the LAR and molecular apocrine gene expression profiles (see Farmer *et al.*, fig. 3A[69] and Lehmann *et al.*, fig. S11[56]). This suggests that gene expression profiling is not just selecting for AR-expressing tumors but a subset of AR-expressing tumors that have an underlying biological signature. Interestingly, the gene profiles of the AR-expressing and AR growth-stimulated triple negative cell lines mentioned above all corresponded to the LAR subtype,[56] suggesting the currently available AR-expressing triple negative cell lines available might not be representative of the full spectrum of AR-expressing TNBC. Therefore, it is important to note that AR immunoreactivity in TNBC specimens does not necessarily indicate that these cases correspond to the androgen-enriched (LAR, molecular apocrine) subtypes of TNBC. Therefore, the possibility for divergent effects of AR action in TNBC could account for the current apparent contradictions in published reports between gene expression profile studies and clinical pathology studies. Further research into potential sub-subtyping of AR-expressing TNBC, including assessment of additional steroid receptors known to activated by androgen derivatives such as ERβ, may help to clarify the underlying biology.

In conclusion, results of our present study suggest that AR and androgen signalling pathways within the tumor may be beneficial to the clinical outcome of TNBC breast cancer patients through the inhibition of cellular proliferation. If borne out by further investigations, this could provide a subset of TNBC patients access to more targeted therapies than those currently available.

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Disclosure Statement

The authors have no conflicts of interest.

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References

1 Youlden DR, Cramb SM, Dunn NA, Muller JM, Pyke CM, Baade PD. The descriptive epidemiology of female breast cancer: an international comparison of screening, incidence, survival and mortality. *Cancer Epidemiol* 2012; **36**: 237–48.
2 Maxmen A. The hard facts. *Nature* 2012; **485**: S50–1.
3 Parise CA, Bauer KR, Brown MM, Caggiano V. Breast cancer subtypes as defined by the estrogen receptor (ER), progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2) among women with invasive breast cancer in California, 1999–2004. *Breast J* 2009; **15**: 593–602.
4 Meijnen P, Peterse JL, Antonini N, Rutgers EJ, van de Vijver MJ. Immunohistochemical categorisation of ductal carcinoma in situ of the breast. *Br J Cancer* 2008; **98**: 137–42.
5 Hanley K, Wang J, Bourne P *et al.* Lack of expression of androgen receptor may play a critical role in transformation from in situ to invasive basal subtype of high-grade ductal carcinoma of the breast. *Hum Pathol* 2008; **39**: 386–92.
6 Wei B, Wang J, Bourne P *et al.* Bone metastasis is strongly associated with estrogen receptor-positive/progesterone receptor-negative breast carcinomas. *Hum Pathol* 2008; **39**: 1809–15.
7 Ogawa Y, Hai E, Matsumoto K *et al.* Androgen receptor expression in breast cancer: relationship with clinicopathological factors and biomarkers. *Int J Clin Oncol* 2008; **13**: 431–5.
8 Gonzalez-Angulo AM, Stemke-Hale K, Palla SL *et al.* Androgen receptor levels and association with PIK3CA mutations and prognosis in breast cancer. *Clin Cancer Res* 2009; **15**: 2472–8.
9 Park S, Koo J, Park HS *et al.* Expression of androgen receptors in primary breast cancer. *Ann Oncol* 2010; **21**: 488–92.
10 Niemeier LA, Dabbs DJ, Beriwal S, Striebel JM, Bhargava R. Androgen receptor in breast cancer: expression in estrogen receptor-positive tumors and in estrogen receptor-negative tumors with apocrine differentiation. *Mod Pathol* 2010; **23**: 205–12.
11 Luo X, Shi YX, Li ZM, Jiang WQ. Expression and clinical significance of androgen receptor in triple negative breast cancer. *Chin J Cancer* 2010; **29**: 585–90.
12 Masuda H, Masuda N, Kodama Y *et al.* Predictive factors for the effectiveness of neoadjuvant chemotherapy and prognosis in triple-negative breast cancer patients. *Cancer Chemother Pharmacol* 2011; **67**: 911–7.
13 Aleskandarany MA, Rakha EA, Ahmed MA, Powe DG, Ellis IO, Green AR. Clinicopathologic and molecular significance of phospho-Akt expression in early invasive breast cancer. *Breast Cancer Res Treat* 2011; **127**: 407–16.
14 Micello D, Marando A, Sahnane N, Riva C, Capella C, Sessa F. Androgen receptor is frequently expressed in HER2-positive. ER/PR-negative breast cancers. *Virchows Arch* 2010; **457**: 467–76.
15 Koo JS, Jung W, Jeong J. The predictive role of E-cadherin and androgen receptor on in vitro chemosensitivity in triple-negative breast Cancer. *Jpn J Clin Oncol* 2009; **39**: 560–8.
16 Pristauz G, Petru E, Stacher E *et al.* Androgen receptor expression in breast cancer patients tested for BRCA1 and BRCA2 mutations. *Histopathology* 2010; **57**: 877–84.
17 Chen J, Zhang X, Tian R *et al.* Expression of androgen receptor in breast carcinoma and its relationship with estrogen receptor, progesterone receptor and HER2 status. *Zhonghua Bing Li Xue Za Zhi* 2010; **39**: 743–6.
18 Chae BJ, Lee A, Bae JS, Song BJ, Jung SS. Expression of nuclear receptor DAX-1 and androgen receptor in human breast cancer. *J Surg Oncol* 2011; **103**: 768–72.
19 He J, Peng R, Yuan Z *et al.* Prognostic value of androgen receptor expression in operable triple-negative breast cancer: a retrospective analysis based on a tissue microarray. *Med Oncol* 2011; **29**: 406–10.
20 Park S, Koo JS, Kim MS *et al.* Androgen receptor expression is significantly associated with better outcomes in estrogen receptor-positive breast cancers. *Ann Oncol* 2011; **22**: 1755–62.
21 Tang D, Xu S, Zhang Q, Zhao W. The expression and clinical significance of the androgen receptor and E-cadherin in triple-negative breast cancer. *Med Oncol* 2011; **29** (2): 526–33.
22 Loibl S, Muller BM, von Minckwitz G *et al.* Androgen receptor expression in primary breast cancer and its predictive and prognostic value in patients treated with neoadjuvant chemotherapy. *Breast Cancer Res Treat* 2011; **130**: 477–87.
23 Qi JP, Yang YL, Zhu H *et al.* Expression of the androgen receptor and its correlation with molecular subtypes in 980 chinese breast cancer patients. *Breast Cancer (Auckl)* 2012; **6**: 1–8.
24 Irshad S, Ellis P, Tutt A. Molecular heterogeneity of triple-negative breast cancer and its clinical implications. *Curr Opin Oncol* 2011; **23**: 566–77.
25 Perou CM. Molecular stratification of triple-negative breast cancers. *Oncologist* 2011; **16**(Suppl. 1): 61–70.
26 Weigman VJ, Chao HH, Shabalin AA *et al.* Basal-like Breast cancer DNA copy number losses identify genes involved in genomic instability, response to therapy, and patient survival. *Breast Cancer Res Treat* 2012; **133**: 865–80.
27 Bauer K, Parise C, Caggiano V. Use of ER/PR/HER2 subtypes in conjunction with the 2007 St Gallen Consensus Statement for early breast cancer. *BMC Cancer* 2010; **10**: 228.
28 Schwentner L, Wolters R, Koretz K *et al.* Triple-negative breast cancer: the impact of guideline-adherent adjuvant treatment on survival-a retrospective multi-centre cohort study. *Breast Cancer Res Treat* 2011; **132**: 1073–80.
29 Razzak AR, Lin NU, Winer EP. Heterogeneity of breast cancer and implications of adjuvant chemotherapy. *Breast Cancer* 2008; **15**: 31–4.
30 Carey LA. Directed therapy of subtypes of triple-negative breast cancer. *Oncologist* 2011; **16**(Suppl. 1): 71–8.
31 Bosch A, Eroles P, Zaragoza R, Vina JR, Lluch A. Triple-negative breast cancer: molecular features, pathogenesis, treatment and current lines of research. *Cancer Treat Rev* 2010; **36**: 206–15.
32 Peiro G, Adrover E, Sanchez-Tejada L *et al.* Increased insulin-like growth factor-1 receptor mRNA expression predicts poor survival in immunophenotypes of early breast carcinoma. *Mod Pathol* 2011; **24**: 201–8.
33 Nogi H, Kobayashi T, Suzuki M *et al.* EGFR as paradoxical predictor of chemosensitivity and outcome among triple-negative breast cancer. *Oncol Rep* 2009; **21**: 413–7.
34 Rennstam K, McMichael N, Berglund P *et al.* Numb protein expression correlates with a basal-like phenotype and cancer stem cell markers in primary breast cancer. *Breast Cancer Res Treat* 2010; **122**: 315–24.
35 Smid M, Hoes M, Sieuwerts AM *et al.* Patterns and incidence of chromosomal instability and their prognostic relevance in breast cancer subtypes. *Breast Cancer Res Treat* 2011; **128**: 23–30.
36 Hussein YR, Sood AK, Bandyopadhyay S *et al.* Clinical and biological relevance of enhancer of zeste homolog 2 in triple-negative breast cancer. *Hum Pathol* 2011; **43**: 1638–44.
37 Svoboda M, Sana J, Redova M *et al.* MiR-34b is associated with clinical outcome in triple-negative breast cancer patients. *Diagn Pathol* 2012; **7**: 31.
38 Moinfar F, Okcu M, Tsybrovskyy O *et al.* Androgen receptors frequently are expressed in breast carcinomas: potential relevance to new therapeutic strategies. *Cancer* 2003; **98**: 703–11.
39 Hall RE, Aspinall JO, Horsfall DJ *et al.* Expression of the androgen receptor and an androgen-responsive protein, apolipoprotein D, in human breast cancer. *Br J Cancer* 1996; **74**: 1175–80.
40 Isola JJ. Immunohistochemical demonstration of androgen receptor in breast cancer and its relationship to other prognostic factors. *J Pathol* 1993; **170**: 31–5.
41 Kuenen-Boumeester V, Van der Kwast TH, van Putten WL, Claassen C, van Ooijen B, Henzen-Logmans SC. Immunohistochemical determination of androgen receptors in relation to oestrogen and progesterone receptors in female breast cancer. *Int J Cancer* 1992; **52**: 581–4.
42 Lea OA, Kvinnsland S, Thorsen T. Improved measurement of androgen receptors in human breast cancer. *Cancer Res* 1989; **49**: 7162–7.
43 Hu R, Dawood S, Holmes MD *et al.* Androgen receptor expression and breast cancer survival in postmenopausal women. *Clin Cancer Res* 2011; **17**: 1867–74.
44 Tsutsumi Y. Apocrine Carcinoma as Triple-negative Breast Cancer: novel Definition of Apocrine-type Carcinoma as Estrogen/Progesterone Receptor-negative and Androgen Receptor-positive Invasive Ductal Carcinoma. *Jpn J Clin Oncol* 2012; **42**: 375–86.
45 McNamara K, Yoda T, Takagi K, Miki Y, Suzuki T, Sasano H. Androgen receptor in triple negative breast cancer. *J Steroid Biochem Mol Biol* 2013; **133**: 66–76.
46 Agoff SN, Swanson PE, Linden H, Hawes SE, Lawton TJ. Androgen receptor expression in estrogen receptor-negative breast cancer. Immunohistochemical, clinical, and prognostic associations. *Am J Clin Pathol* 2003; **120**: 725–31.
47 Rakha EA, El-Sayed ME, Green AR, Lee AH, Robertson JF, Ellis IO. Prognostic markers in triple-negative breast cancer. *Cancer* 2007; **109**: 25–32.
48 Peters AA, Buchanan G, Ricciardelli C *et al.* Androgen receptor inhibits estrogen receptor-alpha activity and is prognostic in breast cancer. *Cancer Res* 2009; **69**: 6131–40.
49 Ormandy CJ, Clarke CL, Kelly PA, Sutherland RL. Androgen regulation of prolactin-receptor gene expression in MCF-7 and MDA-MB-453 human breast cancer cells. *Int J Cancer* 1992; **50**: 777–82.
50 Hall RE, Tilley WD, McPhaul MJ, Sutherland RL. Regulation of androgen receptor gene expression by steroids and retinoic acid in human breast-cancer cells. *Int J Cancer* 1992; **52**: 778–84.
51 Birrell SN, Bentel JM, Hickey TE *et al.* Androgens induce divergent proliferative responses in human breast cancer cell lines. *J Steroid Biochem Mol Biol* 1995; **52**: 459–67.
52 Bentel JM, Birrell SN, Pickering MA, Holds DJ, Horsfall DJ, Tilley WD. Androgen receptor agonist activity of the synthetic progestin, medroxyprogesterone acetate, in human breast cancer cells. *Mol Cell Endocrinol* 1999; **154**: 11–20.

53 Mitchell S, Abel P, Madaan S *et al.* Androgen-dependent regulation of human MUC1 mucin expression. *Neoplasia* 2002; **4**: 9–18.
54 Doane AS, Danso M, Lal P *et al.* An estrogen receptor-negative breast cancer subset characterized by a hormonally regulated transcriptional program and response to androgen. *Oncogene* 2006; **25**: 3994–4008.
55 Robinson JL, MacArthur S, Ross-Innes CS *et al.* Androgen receptor driven transcription in molecular apocrine breast cancer is mediated by FoxA1. *EMBO J* 2011; **30**: 3019–27.
56 Lehmann BD, Bauer JA, Chen X *et al.* Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Invest* 2011; **121**: 2750–67.
57 Ni M, Chen Y, Lim E *et al.* Targeting androgen receptor in estrogen receptor-negative breast cancer. *Cancer Cell* 2011; **20**: 119–31.
58 Gucalp A, Traina TA. Triple-negative breast cancer: role of the androgen receptor. *Cancer J* 2010; **16**: 62–5.
59 Suzuki T, Miki Y, Moriya T *et al.* 5α-reductase type 1 and aromatase in breast carcinoma as regulators of in situ androgen production. *Int J Cancer* 2007; **120**: 285–91.
60 Chanplakorn N, Chanplakorn P, Suzuki T *et al.* Increased 5alpha-reductase type 2 expression in human breast carcinoma following aromatase inhibitor therapy: the correlation with decreased tumor cell proliferation. *Horm Cancer* 2011; **2**: 73–81.
61 McCarty KS Jr, Szabo E, Flowers JL *et al.* Use of a monoclonal anti-estrogen receptor antibody in the immunohistochemical evaluation of human tumors. *Cancer Res* 1986; **46**: 4244s–8s.
62 Sasano H, Suzuki T, Miki Y, Moriya T. Intracrinology of estrogens and androgens in breast carcinoma. *J Steroid Biochem Mol Biol* 2008; **108**: 181–5.
63 Garay JP, Karakas B, Abukhdeir AM *et al.* The growth response to androgen receptor signaling in ERalpha-negative human breast cells is dependent on p21 and mediated by MAPK activation. *Breast Cancer Res* 2012; **14**: R27.
64 Hackenberg R, Hawighorst T, Filmer A *et al.* Regulation of androgen receptor mRNA and protein level by steroid hormones in human mammary cancer cells. *J Steroid Biochem Mol Biol* 1992; **43**: 599–607.
65 Miyashita M, Ishida T, Ishida K *et al.* Histopathological subclassification of triple negative breast cancer using prognostic scoring system: five variables as candidates. *Virchows Arch* 2011; **458**: 65–72.
66 Grynberg M, Fanchin R, Dubost G *et al.* Histology of genital tract and breast tissue after long-term testosterone administration in a female-to-male transsexual population. *Reprod Biomed Online* 2010; **20**: 553–8.
67 Stute P, Sielker S, Wood CE *et al.* Life stage differences in mammary gland gene expression profile in non-human primates. *Breast Cancer Res Treat* 2012; **133**: 617–34.
68 Hackenberg R, Luttchens S, Hofmann J, Kunzmann R, Holzel F, Schulz KD. Androgen sensitivity of the new human breast cancer cell line MFM-223. *Cancer Res* 1991; **51**: 5722–7.
69 Farmer P, Bonnefoi H, Becette V *et al.* Identification of molecular apocrine breast tumours by microarray analysis. *Oncogene* 2005; **24**: 4660–71.
70 Holliday DL, Speirs V. Choosing the right cell line for breast cancer research. *Breast Cancer Res* 2011; **13**: 215.
71 Mehta JP, O'Driscoll L, Barron N, Clynes M, Doolan P. A microarray approach to translational medicine in breast cancer: how representative are cell line models of clinical conditions? *Anticancer Res* 2007; **27**: 1295–300.
72 Ross DT, Perou CM. A comparison of gene expression signatures from breast tumors and breast tissue derived cell lines. *Dis Markers* 2001; **17**: 99–109.
73 Vranic S, Gatalica Z, Wang ZY. Update on the molecular profile of the MDA-MB-453 cell line as a model for apocrine breast carcinoma studies. *Oncol Lett* 2011; **2**: 1131–7.
74 Capes-Davis A, Theodosopoulos G, Atkin I *et al.* Check your cultures! A list of cross-contaminated or misidentified cell lines. *Int J Cancer* 2010; **127**: 1–8.
75 Moore NL, Buchanan G, Harris J *et al.* An androgen receptor mutation in the MDA-MB-453 cell line model of molecular apocrine breast cancer compromises receptor activity. *Endocr Relat Cancer* 2012; **19** (4): 599–613.
76 Sorlie T, Perou CM, Tibshirani R *et al.* Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA* 2001; **98**: 10869–74.
77 Sorlie T, Tibshirani R, Parker J *et al.* Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci USA* 2003; **100**: 8418–23.
78 Perou CM, Sorlie T, Eisen MB *et al.* Molecular portraits of human breast tumours. *Nature* 2000; **406**: 747–52.

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Original Article

Immunohistochemical analysis of aromatase in metastatic lymph nodes of breast cancer

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Aromatase is the key enzyme in intratumoral estrogen production in postmenopausal breast cancer and third generation aromatase inhibitors suppress this enzymatic reaction effectively. Aromatase inhibitor is administered to metastatic breast cancer patients customarily in which estrogen receptor had been demonstrated only in the primary tumor, not the metastatic sites. The status of aromatase in metastatic sites has not been well-characterized to date. We immunolocalized aromatase in 46 estrogen receptor positive primary breast cancers and paired metastatic lymph nodes, using immunohistochemistry. Immunoreactivity was detected in 44/46 primary tumors and 40/46 metastatic lymph nodes. A significant correlation was detected between the status of aromatase in primary and metastatic sites. Aromatase immunoreactivity was correlated with age, size of primary tumor and Ki-67 index. Aromatase immunoreactivity was also detected in adipose tissue surrounding the lymph nodes. In conclusion, aromatase status in primary tumors generally represents its status in metastatic lymph nodes. This indicates that the endocrine environment of estrogen receptor positive tumors remain stable during the metastatic process.

Key words: aromatase, breast cancer, endocrinology, immunohistochemistry, nodal metastasis

Recent gene expression studies of breast cancer identified molecularly distinct subtypes based on intrinsic gene sets.[1] Routine immunohistochemical analysis of estrogen receptor (ER), progesterone receptor (PgR), HER2 and Ki-67 can be used to define the major breast cancer subtypes; luminal A (ER or PgR+, HER2–), luminal B (ER or PgR+, HER2+ or Ki-67 >14%), HER2-overexpression (ER and PgR–, HER2+), triple-negative (ER and PgR–, HER2–).[2] The most frequent types of breast cancer are ER+ tumors or luminal A/B types. Third generation aromatase inhibitors (AIs) have become the gold standard of endocrine therapy for luminal types, by suppressing aromatase, the key enzyme in estrogen biosynthesis. They are reported to elongate disease free survival and decrease recurrence rates compared to conventional endocrine therapy.[3–6] However, the truth remains that despite potent estrogen inhibition by AI treatment, many ER+ tumors eventually metastasize and develop resistance to AIs.

When tumors metastasize, biological status of primary tumors is considered to be maintained in the metastasized tumor cells; therefore, aromatase expressed in tumor cells and the surrounding stromal cells at primary tumors,[7] should also be preserved at metastatic tumor foci and its surrounding stromal cells. Several investigators, however, have reported that the ER status in metastatic lymph nodes was decreased compared to that in primary tumors,[8,9] which raises a question as to whether aromatase status in the metastatic foci is indeed the same as in the primary tumor in the same patients.

We hypothesized that the aromatase status in metastasized lymph nodes would be different from the status in primary tumors, which could explain why breast cancer acquires resistance during its process of progression. Therefore, we compared aromatase immunoreactivity in primary tumors and paired metastasized lymph nodes of breast cancer in the same patients. We also correlated the findings with clinicopathological factors and intrinsic subtype of individual patients.

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MATERIAL AND METHODS

Patients

The patients with breast cancer examined in our study were all operated on at Tohoku University Hospital, Sendai, Japan, and were diagnosed with pure invasive ductal carcinoma with lymph node metastases. 10% formalin fixed and paraffin embedded tissue sections from both the primary tumors and the corresponding lymph node metastases were screened among 123 cases diagnosed with positive lymph node metastases from 2004 to 2008. The patients who had distant metastases or had received neoadjuvant chemotherapy or radiotherapy at the time of operation were subsequently excluded from this study. In addition, in seven cases, the lymph node metastatic sites were diminished while making multiple tissue sections so these cases were also excluded from the present study. Therefore, a total of 46 formalin-fixed, paraffin embedded primary tumor tissues and paired lymph nodes were available for this study. Clinicopathological features of the cases examined are summarized in Table 1. Research protocols for this study were approved by the ethics committee at Tohoku University School of Medicine (2010-570).

Table 1 Patients and tumor characteristics

Patient characteristics	*N*	%
Age (Years): Median 56, Mean 58.1 (Range 41–82)		
≥40	2	4.3
41–50	9	19.6
51–60	17	37.0
61–70	8	17.4
≥71	10	21.7
pTNM stage		
II	25	54.3
III	21	45.7
IV	0	0.0
Primary tumor size (cm)		
≤2	20	43.5
2–5	25	54.3
>5	1	2.2
Tumor grade		
1	12	26.1
2	20	43.5
3	14	30.4
Pathological type		
Scirrhous	31	67.4
Papillo-tubular	6	13.0
Solid-tubular	7	15.2
Mucinous	1	2.2
IMPCa	3	6.5
Number of lymph nodes with metastasis		
1–3 (pN1)	29	63.0
4–9 (pN2)	13	28.3
≥10 (pN3)	4	8.7

All patients were diagnosed with ER positive invasive ducal carcinoma.

Immunohistochemistry

The characteristics of primary antibody for aromatase (aromatase monoclonal antibody 677)[10] were previously reported by Miki *et al.* Briefly, after deparaffinization, sections were washed with PBS and treated with 0.3% hydrogen peroxidase in methanol for 20 min. Normal rabbit serum (1%) was applied to the sections for 20 min and primary antibody was applied to the tissue sections for 18 h at 4C. Reacted sections were subsequently incubated with anti-mouse immunoglobulin for 20 min, followed by exposure to peroxidase-conjugated streptavidin for 20 min at room temperature. Immunoreactivity was detected by immersing the tissue sections in 3,30-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM tris–HCl buffer (pH 7.6), 10 mM sodium azide, 0.006% hydrogen peroxidase). Sections were counterstained with hematoxylin.

Other antibodies used in this study were as follows: ERalpha (ER1D5; Immunotech S.A., Marseilles, France), PgR (MAB429; Chemicon International Inc., Temecula, CA, USA), HER2 and Ki-67 (MIB1; DakoCytomation Co. Ltd, Kyoto, Japan).

A Histofine Kit (Nichirei, Tokyo, Japan) was used for immunohistochemistry in our study. The antigen–antibody complex was visualized with 3, 3′-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris–HCl buffer (pH 7.6), and 0.006% H2O2) and counterstained with hematoxylin.

Evaluation of immunohistochemistry

Aromatase immunoreactivity was detected in the cytoplasm. The approximate percentage of stained cells (proportion score) were classified into the following four groups: 0, <1%; 1, –25%; 2, –50%; and 3, >50%. Relative intensity of aromatase immunopositive cells was classified as follows: 0, no immunoreactivity; 1, weak; 2, moderate; and 3, intense immunoreactivity, according to the report by Miki *et al.*[10] Aromatase immunoreactivity was evaluated as a total score of proportion score and intensity score.[10]

Immunoreactivity of ER and PgR was detected in the nuclei of cancer cells. A number of positive cells were counted in 10 random optic fields, using a light microscope equipped with 50x objective lenses. In small lymph node metastatic sites where 10 optic fields were unavailable, all cancer cells were evaluated. Subsequently, the percentage of immunoreactivity, i.e. labeling index (LI), was determined.[11] The ER and PgR LI of more than 10% were considered positive.[12] The ER and PgR were also scored for Allred score (0–8).

HER2 immunoreactivity was detected at the membrane of cancer cells and was evaluated according to the grading system (HercepTest 0, 1+, 2+, 3+)(DAKO).

Ki-67 immunoreactivity was detected in the nuclei and evaluated using labeling index (LI). More than 10% of positive tumor cells were tentatively considered positive staining[13]

Statistical analysis.

All the statistical analysis was performed using StatView (SAS Institute, San Francisco, CA, USA). A *P-value* of <0.05 was considered statistically significant.

RESULTS

Aromatase immunoreactivity

A total of 44 PTs (44/46, 95.7%) were immunohistochemically positive for aromatase. Among them, 36 cases (36/46, 78.3%) demonstrated aromatase immunoreactivity in more than 50% of the tumor cells (proportion score (PS)3). Aromatase immunoreactivity was evaluated in matched metastasized lymph nodes (metastatic LNs) of those 46 patients above. Aromatase immunoreactivity in lymph nodes were detected in 40 cases (40/46, 87.0%), and among these 40 cases, 35 cases (35/46, 76.1%) were in PS3 status. The mean percentage of immunopositive cells was 71.2% in PT and 65.7% in metastatic LNs. Aromatase immunoreactivity of tumor cells in PTs and the paired metastatic LNs demonstrated good concordance (Y = 44.494 + 0.298 *X; R^2 = 0.067, $P < 0.05$) using regression analysis. The results of aromatase immunohistochemical scores are summarized in Figure 1.

Among 41 PTs associated with a high proportion score (PS3 or 2) in PTs, 35 cases also demonstrated high PS in metastatic LNs while only six cases showed low PS (PS1 or 0). However, four out of five cases with low PS scores in PTs were associated with increased PS scores in metastatic LNs. In addition, 10 out of 42 cases with high scores in PTs were associated with low intensity scores (IS) in metastatic LNs, whilst three out of four cases with low IS in PTs demonstrated high scores in metastatic LNs. Seven (15.2%), 22 (47.8%), 17 (37.0%) of the cases demonstrated increment, no-changes and decrement of PS, respectively (Fig. 1b), and 35 (76.1%) and 11 (23.9%) cases were associated with increased and decreased IS, respectively (Fig. 1a). In total score (PS + IS), 9 (19.6%), 21 (45.7%) and 16 (34.8%) cases were associated with increment, no changes and decrement in metastatic LNs compared to PTs, respectively (Fig. 1c). Representative illustrations of staining patterns for PTs and the corresponding metastatic LNs (which both were scored as 3+) were illustrated in Figure 2.

Aromatase immunoreactivity was detected in stromal components of PTs as well as metastatic LNs, including fibroblasts and endothelial cells. Relatively weak immunoreactivity was also detected in adjacent lymphocytes in metastasized lymph nodes as well as non-metastasized lymph nodes. The adjacent lymphoid follicles of metastatic LNs were negative for aromatase, as well as the non-metastasized lymph nodes (Fig. 3).

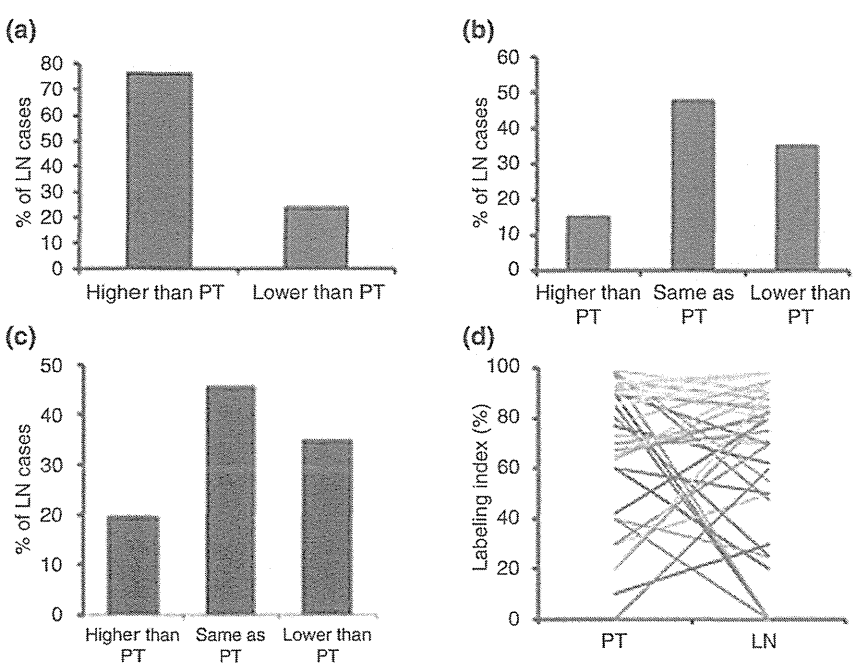

Figure 1 Aromatase expression changes in metastasized lymph nodes (LN) compared to matched primary tumor (PT). (**a**) Proportion score, (**b**) Intensity score, (**c**) Total score, (**d**) Labeling index (%).

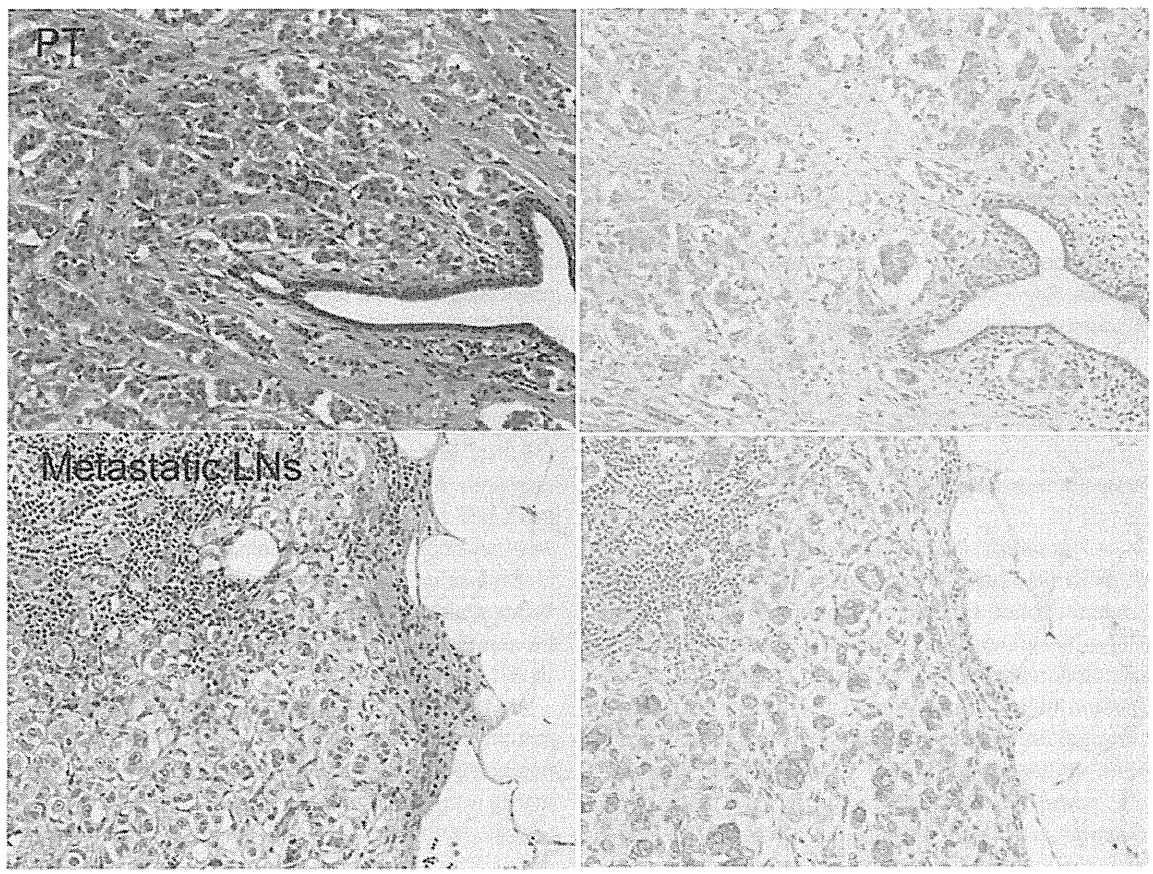

Figure 2 Hematoxylin-Eosin staining (left) and immunohistochemical staining of aromatase (right) in primary tumor (upper) and paired metastasized lymph node (lower). ×10 magnification. This patient was 51 years old post-menopausal female, diagnosed as infiltrating ductal carcinoma. Immunohistochemically, the tumor was ER, PgR positive and HER2 negative. For aromatase staining, primary tumor was scored PS3 + IS3 = TS6; lymph node was also scored PS3 + IS3 = TS6.

Correlation of aromatase in PT and metastatic LNs with histopathological parameters

Aromatase immunoreactivity in PT was significantly correlated with a small PT size (aromatase cut off value 50% and 75%, $P < 0.05$) and size of largest metastatic LNs (aromatase cut off values 50 and 75%, $P < 0.05$). Aromatase immunoreactivity in metastatic LNs was negatively correlated with Ki-67 labeling index in PTs (aromatase cut off value 25%, $P = 0.0197$) and aromatase immunoreactivity in metastatic LNs with younger age (aromatase cut off value 50 and 75%, age ≤60, $P = 0.0171$ and 0.0009)(Table 2).

Heterogeneous patterns of aromatase status between primary tumor and metastasized lymph nodes

We divided the aromatase staining pattern between PT and metastatic LNs in to two, heterogeneous and homogeneous. Cases which showed a change of more than 2-fold between PT and metastatic LNs in terms of the approximate percentage of aromatase positive cells was tentatively named the 'heterogeneous' group; cases which showed a change of less than 2-fold between PT and metastatic LNs was named the 'homogeneous' group. Eleven cases (11/46, 23.9%) corresponded to 'heterogeneous' group; four increased and seven decreased patterns in metastatic LNs compared to PT. The remaining 35 cases corresponded to 'homogeneous' group. Figures 4 and 5 shows heterogeneous aromatase staining between PT (Fig. 4) and metastatic LNs (Fig. 5) in the same case. The 'heterogeneous' group of the patients was associated with significantly younger age, smaller size of largest metastatic LNs and lower ER status in PTs compared to the 'homogeneous' group of the patients (Table 3).

DISCUSSION

In breast cancer, an interaction of tumor cells with surrounding stromal cells plays a critical role in estrogen production

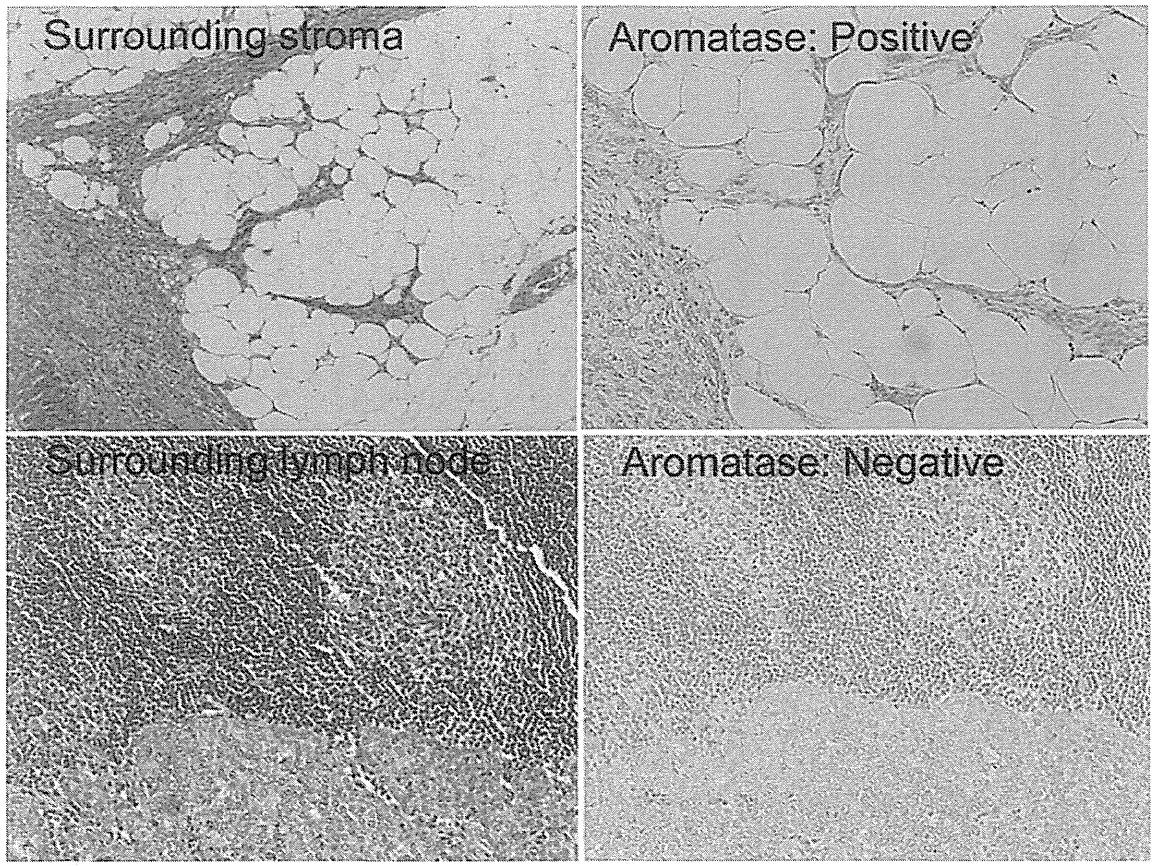

Figure 3 Hematoxylin-Eosin staining (left) and immunohistochemical staining of aromatase (right) in surrounding stroma of metastasized lymph nodes (upper) and adjacent lymphoid follicle of metastasized lymph nodes (lower). ×10 magnification. This patient is the same case used in Figure 2. In the surrounding stroma, we observed immunohistochemical aromatase positivity in the adipose tissue. Its intensity was same as/weaker than tumor cells in metastasized lymph nodes. In the adjacent lymphoid follicle, we did not observe any positivity in the same way we observed positivity in tumor cells.

via the aromatase enzyme.[10] Intratumoral aromatase has been detected in stromal cells such as adipocytes and fibroblasts as well as in parenchymal or cancer cells.[10,14] Reports show that these aromatase positive stromal cells promote the process of invasion and metastasis.[15] Such a model raises the following questions: (i) what is the location of aromatase enzyme expression in the metastasized lymph nodes? and (ii) do tumor cells synthesize aromatase via tumor-stromal interaction at the metastatic sites as in primary tumors?

Aromatase immunoreactivity was recently reported to be decreased in lymph node metastatic sites when compared to the primary sites, using tissue microarray, which they concluded may account for potential resistance to endocrine therapy at metastatic sites.[16] However, it is also known that results of tissue microarray may not be compared to those evaluated in the whole sections, due to the heterogeneous nature of aromatase staining, which calls for investigation using whole sections.[17] Therefore, in our present study, aromatase immunoreactivity was evaluated in paired whole tissue sections of primary breast cancer and the corresponding metastasized lymph nodes in ER+ breast cancer tissues.

Aromatase immunoreactivity was detected in metastatic cancer cells of lymph nodes as well as the surrounding stromal cells or fibroblasts in ER+ breast cancer using whole tissue sections. There was a statistically significant positive correlation between aromatase status in PTs and metastatic LNs; with metastatic LNs demonstrating slightly higher aromatase immunoreactivity compared to PTs. Such consistent patterns of aromatase expression in metastatic LNs indicates that aromatase immunoreactivity in PTs, which can be easily evaluated following surgery or biopsy using immunohistochemistry, can predict aromatase status in metastatic LNs with high probability.

There were, however, cases in which aromatase status between PTs and metastatic LNs were clearly discordant. Interestingly, these cases were associated with significantly lower ER positivity in PTs. Aromatase immunoreactivity of PTs is not a predictive factor of response to endocrine

Table 2 Aromatase protein expression and patient clinicopathological data

		25% Aromatase PT (+), (–)	*P*	25% Aromatase LN (+), (–)	*P*	50% Aromatase PT (+), (–)	*P*	50% Aromatase LN (+), (–)	*P*	75% Aromatase PT (+), (–)	*P*	75% Aromatase LN (+), (–)	*P*
Age	≤55	19, 1	0.3693	18, 2	0.435	19, 1	***0.0278***	15, 5	0.7495	14, 6	0.364	12, 8	ns*
	>55	22, 4		20, 6		17, 9		18, 8		14, 12		15, 11	
	≤60	16, 2	ns*	16, 2	0.4525	14, 4	ns*	9, 9	***0.0171***	7, 11	***0.0288***	5, 13	***0.0009***
	>60	25, 3		22, 6		22, 6		24, 4		21, 7		22, 6	
Histological Grade	1	12, 0	0.3059	11, 1	0.6598	10, 2	ns*	10, 2	0.4614	9, 3	0.3151	9, 3	0.3071
	2&3	29, 5		27, 7		26, 8		23, 11		19, 15		18, 16	
Primary tumour size	<2	16, 2	ns*	15, 3	ns*	15, 3	0.7172	14, 4	0.5223	13, 5	0.2344	14, 4	0.0644
	≥2	25, 3		23, 5		21, 7		19, 9		15, 13		13, 15	
	<2.5	27, 3	ns*	24, 6	0.6942	27, 3	***0.0202***	22, 8	0.7441	23, 7	***0.0043***	19, 11	0.531
	≥2.5	14, 2		14, 2		9, 7		11, 5		5, 11		8, 8	
	<3	32, 2	0.1029	27, 7	0.6598	28, 6	0.4157	23, 11	0.4614	24, 10	***0.0383***	19, 15	0.7346
	≥3	9, 3		11, 1		8, 4		10, 2		4, 8		8, 4	
Number of metastatic LN	1 to 3	26, 3	ns*	22, 7	0.2263	24, 5	0.4623	19, 10	0.315	18, 11	ns*	15, 14	0.2352
	4–	15, 2		16, 1		12, 5		14, 3		10, 7		12, 5	
Size of largest metastatic LN	<1 cm	23, 2	0.6476	19, 6	0.2597	20, 5	ns	15, 10	0.0987	18, 7	0.1317	13, 12	0.3769
	≥1 cm	18, 3		19, 2		16, 5		18, 3		10, 11		14, 7	
	<2 cm	38, 4	0.3794	36, 6	0.1341	35, 7	***0.0278***	32, 10	0.0622	27, 15	***0.0238***	26, 16	0.2916
	≥2 cm	3, 1		2, 2		1, 3		1, 3		0, 4		1, 3	
LN ER status	(+)	40, 5	ns*	38, 7	0.1739	36, 9	0.2174	33, 12	0.2826	28, 17	0.3913	27, 18	0.413
	(–)	1, 0		0, 1		0, 1		0, 1		0, 1		0, 1	
PT PgR status	(+)	28, 4	ns*	27, 5	0.684	25, 7	ns*	23, 9	ns*	22, 10	0.1152	21, 11	0.1988
	(–)	13, 1		11, 3		11, 3		10, 4		6, 8		6, 8	
LN PgR status	(+)	33, 5	0.5692	33, 5	0.1294	30, 8	ns*	28, 10	0.6689	25, 13	0.2316	25, 13	0.0505
	(–)	8, 0		5, 3		6, 2		5, 3		3, 5		2, 6	
PT ER (LI)	<50	5, 0	ns*	5, 0	0.5692	4, 1	ns*	3, 2	0.6119	3, 2	ns*	2, 3	0.6351
	≥50	36, 5		33, 8		32, 9		30, 11		25, 16		25, 16	
PT PgR (LI)	<50	22, 0	0.0502	18, 4	ns*	19, 3	0.2894	17, 5	0.5207	13, 9	ns*	13, 9	ns*
	≥50	19, 5		20, 4		17, 7		16, 8		15, 9		14, 10	
PT HER2	0,1,2	34, 4	ns*	31, 7	ns*	29, 9	0.6641	27, 11	ns*	22, 16	0.4525	21, 17	0.4395
	3	7, 1		7, 1		7, 1		6, 2		6, 2		6, 2	
PT Ki-67 (LI)	≤10	21, 3	ns*	23, 1	***0.0197***	20, 4	0.4839	19, 5	0.3304	16, 8	0.5468	16, 18	0.3695
	>10	20, 2		15, 7		16, 6		14, 8		12, 10		11, 11	
LN Ki-67 (LI)	≤10	16, 1	0.6375	16, 1	0.2263	14, 3	0.7227	14, 3	0.315	9, 8	0.5335	12, 5	0.2352
	>10	25, 4		22, 7		22, 7		19, 10		19, 10		15, 14	

*ns, not significant; PT, primary tumor; LN, metastasized lymph node; LI, labeling index (%).

P, *P*-value. A *P*-value <0.05 was considered statistically significant and those values are printed in italic, above.

Aromatase expression and its correlation with clinic-pathological factors were examined with a cut-off value of 25, 50 and 75% of positively stained tumor cells using 2×2 χ^2 testing.

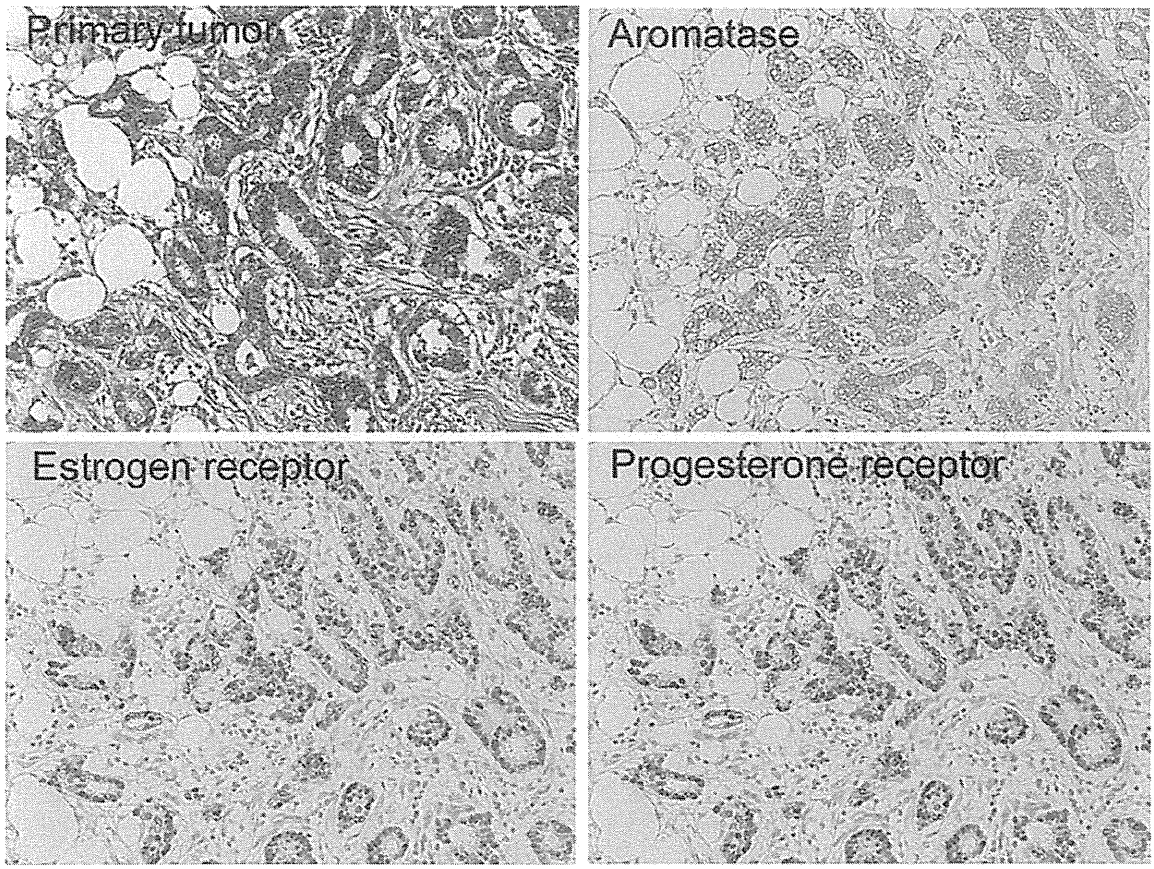

Figure 4 Hematoxylin-Eosin staining (left upper), immunohistochemical staining of estrogen receptor (left lower), progesterone receptor (left lower) and aromatase (right upper) in primary tumor. ×20 magnification. This patient was 61 years old female, diagnosed as infiltrating ductal carcinoma. Immunohistochemically, the primary tumor was ER, PgR positive and HER2 negative. For aromatase staining, the primary tumor was scored PS3 + IS2 = TS5.

therapy[17] but ER positivity in cancer cells is well-known to be a strong predictor of response to endocrine therapy.[18] Therefore, we infer that heterogeneous aromatase expression between PTs and metastatic LNs may be one explanation for variable response to endocrine therapy among ER+ tumors. Also, we noticed a particular result of interest in a few cases which presented with negative aromatase expression in PTs but positive aromatase expression in metastatic LNs. This data suggests a possible indication of aromatase inhibitors in LN metastatic cases with negative aromatase status in PTs. This possibility could only have been discovered by this immunohistochemical analysis of aromatase status in lymph nodes. To clarify this, further study utilizing ER negative breast carcinoma is warranted.

In our present study, a significant negative correlation was detected between the size of PTs and aromatase status of both PTs and metastatic LNs. This result is in good agreement with a prior study by Ellis *et al.*[19] From this, we may hypothesize that aromatase expression decreases as the tumor grows and progresses, not only at primary tumor sites but also at metastasized lymph nodes which could explain this interesting phenomenon. This evidence strengthens the known fact that early detection and treatment of breast cancer is, above all, the most important treatment strategy for breast cancer. In addition, the lower Ki-67 labeling index in PTs was significantly associated with higher aromatase status in metastatic LNs, which suggests that aromatase positive tumor cells may intrinsically have a less aggressive behavior. This finding also implies that for predicting outcomes of ER+ tumors, small tumor size and Ki-67 labeling index in combination with ER, PgR and HER2 status may give the same amount of information that immunohistochemical analysis of aromatase would give. It is therefore suggested that routine immunohistochemical analysis of aromatase in PTs and metastasized LNs would not aid in the prediction of a prognosis in ER+ lymph node positive patients; instead they help clarify the role of aromatase in the process of metastasis.

Our data shows that almost all of the tumors demonstrated ER positivity in metastatic LNs (45/46, 97.8%). This concordance rate is much higher compared to other reported

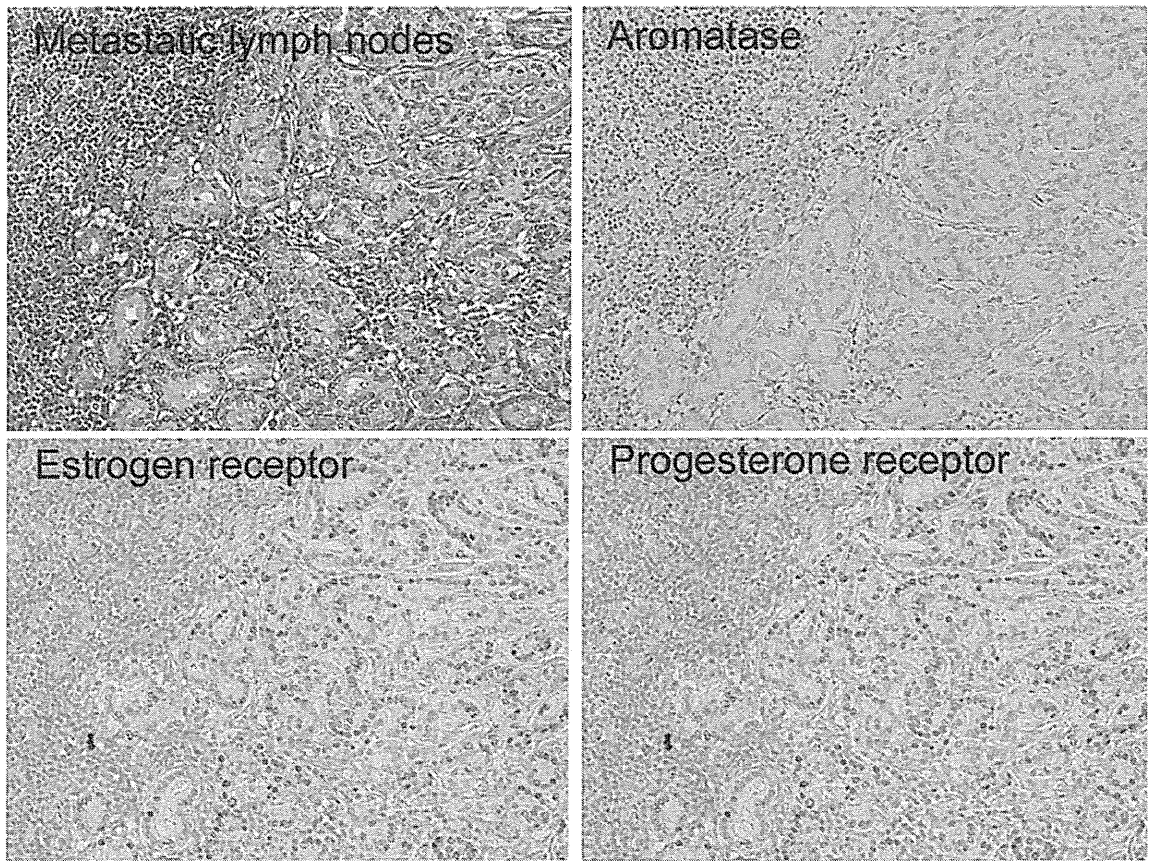

Figure 5 Hematoxylin-Eosin staining (left upper), immunohistochemical staining of estrogen receptor (left lower), progesterone receptor (left lower) and aromatase (right upper) in metastatic lymph nodes. ×10 magnification. This patient is the same patient in Figure 4. Immunohistochemically, the metastasized lymph node was ER, PgR positive as in the primary tumor; however aromatase staining was scored PS0 + IS0 = TS0, negative, which showed heterogenous result between primary tumor and metastasized lymph nodes.

studies,[8] due to the fact that we only examined ER+ tumors in this study. PgR was associated with a more variable result; 10 out of 46 tumors (21.7%) demonstrated disparities between PTs and metastatic LNs. Many previously reported studies have focused on ER expression in PTs and LNs but few have examined thoroughly the discordance of PgR expression between PTs and metastatic LNs. In this study, two out of 32 PgR+ tumors showed conversion to PgR– in metastatic LNs. Of interest, both of these cases were associated with low ER total score (TS5 and 6) and low aromatase expression (0% and 15%). ER+/PgR– breast cancer has been reported to be a distinct subset of breast cancer showing aggressive behavior and endocrine therapy resistance.[20] Therefore, further examination of PgR in metastatic LNs with its relation to prognosis and acquired resistance is warranted.

Heterogeneous aromatase expression patterns among multiple metastatic LNs were observed in this study where 18 out of 34 cases (18/34, 52.9%) with multiple metastatic LNs demonstrated more than a two-fold difference in aromatase immunoreactivity. The cases showing aromatase heterogeneity were significantly correlated with a larger number of metastatic LNs only but also tended to be correlated with weak ER expression in PTs (data not shown). Much of this phenomenon may be explained by the heterogeneous nature of aromatase staining but awaits further examination.

In summary, both cancer cells and surrounding stromal cells at the sites of metastasized lymph nodes generally expressed aromatase in the same manner and the same intensity as in the primary tumor of the same patients with few exceptions. We may conclude that tumor-stromal interaction is as important at the metastasized lymph nodes as it is at the primary tumor in inducing aromatase. These findings demonstrated that once tumor cells get into the lymphatic system, tumors will seed on to lymph nodes to receive interaction from the stromal components of metastatic sites, where aromatase once again synthesizes estrogen for tumor growth. Somehow, some of the tumor cells stop generating aromatase; therefore, source of nutrition lies elsewhere, which warrants further research.

Table 3 Homogeneous and heterogeneous aromatase staining and its correlation with clinicopathological factors

		Homo	Hetero	*P*
Age	≤55	16	4	***0.0232***
	>55	19	7	
	≤60	10	8	***0.0138***
	>60	25	3	
Histological Grade	1	11	1	0.2415
	2&3	24	10	
Primary tumour size	<2	15	3	0.4865
	≥2	20	8	
	<3	27	7	0.4411
	≥3	8	4	
Number of metaLN	1 to 3	21	8	0.5012
	4–	14	3	
Size of largest metaLN	<2 cm	32	10	ns*
	≥2 cm	3	1	
	<1 cm	16	9	***0.0449***
	≥1 cm	19	2	
LN ER status	(+)	34	11	ns*
	(–)	1	0	
PT PgR status	(+)	26	6	0.2691
	(–)	9	5	
LN PgR status	(+)	31	7	0.0789
	(–)	4	4	
PT ER (LI)	<50	6	5	***0.0407***
	≥50	35	6	
PT PgR (LI)	<50	16	6	0.7343
	≥50	19	5	
PT HER2	0,1,2	29	9	ns*
	3	6	2	
PT Ki-67 (LI)	≤10	21	3	0.0861
	>10	14	8	
LN Ki-67 (LI)	≤10	15	2	0.1723
	>10	20	9	

*ns, not significant; *P*, *P*-value. A *P*-value <0.05 was considered statistically significant and those values are printed in bold and italics; above.

Aromatase expression in primary tumors and metastatic lymph nodes were grouped in to two groups.

Homo (homogeneous); change of ≤2-fold, in aromatase expression between PT and metastatic LNs.

Hetero (heterogeneous): change of >2-fold in aromatase expression between PT and metastatic LNs.

PT, primary tumor; LN, metastasized LNs; LI, labeling index (%).

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REFERENCES

1 Sorlie T, Tibshirani R, Parker J *et al.* Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci U S A* 2003; **100**: 8418–23.

2 Perou CM, Sorlie T, Eisen MB *et al.* Molecular portraits of human breast tumours. *Nature* 2000; **406**: 747–52.

3 Joerger M, Thurlimann B. Update of the BIG 1-98 Trial: Where do we stand? *Breast* 2009; **18** Suppl 3: S78–82.

4 Josefsson ML, Leinster SJ. Aromatase inhibitors versus tamoxifen as adjuvant hormonal therapy for oestrogen sensitive early breast cancer in post-menopausal women: Meta-analyses of monotherapy, sequenced therapy and extended therapy. *Breast* 2010; **19**: 76–83.

5 Coombes RC, Kilburn LS, Snowdon CF *et al.* Survival and safety of exemestane versus tamoxifen after 2–3 years' tamoxifen treatment (Intergroup Exemestane Study): A randomised controlled trial. *Lancet* 2007; **369**: 559–70.

6 Mouridsen H, Giobbie-Hurder A, Goldhirsch A *et al.* Letrozole therapy alone or in sequence with tamoxifen in women with breast cancer. *N Engl J Med* 2009; **361**: 766–76.

7 Sasano H, Anderson TJ, Silverberg SG *et al.* The validation of new aromatase monoclonal antibodies for immunohistochemistry—a correlation with biochemical activities in 46 cases of breast cancer. *J Steroid Biochem Mol Biol* 2005; **95**: 35–9.

8 Aitken SJ, Thomas JS, Langdon SP, Harrison DJ, Faratian D. Quantitative analysis of changes in ER, PR and HER2 expression in primary breast cancer and paired nodal metastases. *Ann Oncol* 2010; **21**: 1254–1261.

9 Dikicioglu E, Barutca S, Meydan N, Meteoglu I. Biological characteristics of breast cancer at the primary tumour and the involved lymph nodes. *Int J Clin Pract* 2005; **59**: 1039–44.

10 Miki Y, Suzuki T, Tazawa C *et al.* Aromatase localization in human breast cancer tissues: Possible interactions between intratumoral stromal and parenchymal cells. *Cancer Res* 2007; **67**: 3945–54.

11 Suzuki T, Miki Y, Moriya T *et al.* 5Alpha-reductase type 1 and aromatase in breast carcinoma as regulators of in situ androgen production. *Int J Cancer* 2007; **120**: 285–91.

12 Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol* 1998; **11**: 155–68.

13 D'Andrea MR, Limiti MR, Bari M *et al.* Correlation between genetic and biological aspects in primary non-metastatic breast cancers and corresponding synchronous axillary lymph node metastasis. *Breast Cancer Res Treat* 2007; **101**: 279–84.

14 Sasano H, Nagura H, Harada N, Goukon Y, Kimura M. Immunolocalization of aromatase and other steroidogenic enzymes in human breast disorders. *Hum Pathol* 1994; **25**: 530–35.

15 Liao D, Luo Y, Markowitz D, Xiang R, Reisfeld RA. Cancer associated fibroblasts promote tumor growth and metastasis by modulating the tumor immune microenvironment in a 4T1 murine breast cancer model. *PLoS ONE* 2009; **4**: e7965.

16 Gschwantler-Kaulich D, Fink-Retter A, Czerwenka K *et al.* Differential expression pattern of estrogen receptors, aromatase, and sulfotransferase in breast cancer tissue and corresponding lymph node metastases. *Tumour Biol* 2011; **32**: 501–8.

17 Lykkesfeldt AE, Henriksen KL, Rasmussen BB *et al.* In situ aromatase expression in primary tumor is associated with estrogen receptor expression but is not predictive of response to endocrine therapy in advanced breast cancer. *BMC Cancer* 2009; **9**: 185–96.

18 Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: An overview of the randomised trials. *Lancet* 2005; **365**: 1687–717.

19 Ellis MJ, Miller WR, Tao Y *et al.* Aromatase expression and outcomes in the P024 neoadjuvant endocrine therapy trial. *Breast Cancer Res Treat* 2009; **116**: 371–8.

20 Thakkar JP, Mehta DG. A review of an unfavorable subset of breast cancer: Estrogen receptor positive progesterone receptor negative. *Oncologist* 2011; **16**: 276–85.

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ORIGINAL PAPER

BUB1 Immunolocalization in Breast Carcinoma: Its Nuclear Localization as a Potent Prognostic Factor of the Patients

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Abstract Mitotic checkpoint is a fundamental mechanism involved in fidelity mitotic chromosome segregation, and its alteration results in progression of human malignancies. In this study, we examined expression profiles of seven mitotic checkpoint genes in 20 breast carcinomas using microarray analysis. Results demonstrated that *BUB1* expression level was closely correlated with the proliferation activity evaluated by Ki-67 labeling index (LI) of individual cases. Therefore, we further immunolocalized *BUB1* in 104 breast carcinoma tissues in order to evaluate its clinicopathological significance. *BUB1* immunoreactivity was detected in the nucleus and/or cytoplasm of carcinoma cells, and nuclear and cytoplasmic *BUB1* status were positive in 40% and 58% of the cases examined, respectively. In particular, nuclear *BUB1* status was significantly associated with stage, pathological tumor factors, lymph node metastasis, distant metastasis, histological grade, and Ki-67 LI, but cytoplasmic *BUB1* status was not significantly associated with any of the parameters examined. Subsequent multivariate analysis revealed that nuclear *BUB1* status turned out an independent prognostic factor for both disease-free and breast cancer-specific survival of the patients examined. These results all indicated that *BUB1* played important roles in the proliferation and/or progression of the breast carcinoma, and nuclear *BUB1* immunohistochemical status is also considered a potent prognostic factor in human breast cancer patients.

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Introduction

Breast cancer is one of the most common malignancies in women. Invasive breast cancer is generally regarded as a disease that metastasizes in an early phase [1], and clinical outcome of the patients is markedly influenced not only by metastasis but also by proliferative activity of the carcinoma cells [2, 3]. A multitude of prognostic factors identified in breast cancer patients have been demonstrated to be directly or indirectly correlated with carcinoma cell proliferation.

Cell proliferation is closely associated with altered regulation of the cell cycle [4]. Progression of the cell cycle is regulated by three major checkpoint mechanisms, i.e., G1/S, G2/M, and mitotic checkpoints, which subsequently ensure that each step takes place only once and in the right sequence [5]. Among these factors, the mitotic checkpoint, also known as spindle assembly checkpoint, is to ensure accurate chromosome segregation by inducing mitotic arrest when errors occur in the spindle structure or in the alignment of the chromosomes on the spindle formation [6]. Defective mitotic checkpoint genes have been reported to be implicated as one of the mechanisms of chromosomal instability [5], but significance of alternations of mitotic checkpoint themselves have remained largely unknown in human cancer tissues compared with other checkpoints. Genomic studies in mammals implicated at least seven

genes including *BUB1*, *BUB1B* (*BUBR1* or *MAD3*), *BUB3*, *MAD1*, *MAD2*, *CDC20*, and *TTK* (*MPS1*) [5, 7, 8] in the mitotic checkpoint. Therefore, in this study, we first evaluated expression profiles of mitotic checkpoint genes in the breast carcinoma based on microarray data and did demonstrate that *BUB1* expression level was closely correlated with the proliferative activity of carcinoma cells.

BUB1 is also well-known as a key component of mitotic checkpoint. *BUB1* mutations were originally reported in a subset of aneuploid colorectal carcinoma cell lines [9], suggesting that low expression of *BUB1* could contribute to defective mitotic checkpoint control in human malignancies. However, subsequent studies in various human cancer tissues demonstrated that the mutations of *BUB1* were extremely rare or not detected at all [10–13]. However, Yuan et al. [14] reported that both mRNA and protein levels for mitotic checkpoint genes including *BUB1* were significantly higher in the breast carcinoma cell lines than normal mammary epithelial cells. In addition, Shigeishi et al. [15] reported a positive significant correlation between *BUB1* expression levels and proliferative activity in the salivary gland tumors. These findings all indicated that *BUB1* plays important roles in the proliferation and/or progression of the breast carcinoma. However, *BUB1* immunolocalization has been reported only in the gastric cancer among human malignancies [16] to the best of our knowledge, and clinical significance of *BUB1* has remained unknown in the breast carcinoma. Therefore, in this study, we immunolocalized *BUB1* in human breast cancer tissues in order to clarify its clinicopathological significance.

Materials and Methods

Patients and Tissues

Two sets of tissue specimens were evaluated in this study. As a first set, 20 specimens of invasive breast carcinoma were obtained from women (age, 40–74 years) who underwent surgical treatment from 2000 to 2003 in the Department of Surgery, Tohoku University Hospital, Sendai, Japan. These cases were all estrogen receptor (ER)-positive breast carcinoma patients, and the percentage of ER-positive carcinoma cells (i.e., ER labeling index (LI)) was 4–95% in these cases [17]. These specimens were kept both at−80°C for microarray analysis and fixed in 10% formalin and embedded in paraffin wax for immunohistochemistry for Ki-67.

As a second set, 104 specimens of invasive breast carcinoma were obtained from Japanese female patients who underwent surgical treatment from 1988 to 1999 in the Department of Surgery, Tohoku University Hospital, Sendai, Japan. The mean age of these patients was 55 (range, 22–81 years), and these patients did not receive chemotherapy, irradiation, or hormonal therapy prior to the surgery. Review of the charts revealed that 79 patients received adjuvant chemotherapy and 69 patients received tamoxifen therapy following the surgery. The clinical outcome was evaluated by disease-free and breast cancer-specific survival of the stages I–III patients in this study, and the mean follow-up time was 95 (range, 0–175 months). All the specimens were fixed in 10% formalin and embedded in paraffin wax.

Research protocols for this study were approved by the Ethics Committee at Tohoku University School of Medicine.

Laser Capture Microdissection/Microarray Analysis

Gene expression profiles of laser capture microdissection samples in 20 invasive breast carcinoma cases were examined using microarray analysis. A part of gene expression profile data was assembled in our previous study [18, 19]. Briefly, frozen-specimens of the breast carcinoma were sectioned at a thickness of 8 μm; approximately 5,000 breast carcinoma cells were laser-transferred, and total RNA was extracted. Sample preparation and processing were performed as described in the Affymetrix GeneChip Expression Analysis Manual (Affymetrix), with the exception that the labeled cRNA samples were hybridized to the complete human U133 GeneChip set (Affymetrix), including 22,215 and 22,577 genes. We focused on expression of seven representative mitotic checkpoint genes in this study.

Immunohistochemistry

Rabbit polyclonal antibodies for human *BUB1* (LS-C118685) and γ-tubulin (GTX115850) were purchased from LifeSpan BioSciences (Seattle, WA, USA) and GeneTex (Irvine, CA, USA), respectively. Monoclonal antibodies for ER (ER1D5), progesterone receptor (PR; MAB429), and Ki-67 (MIB1) were purchased from Immunotech (Marseille, France), Chemicon (Temecula, C, USA), and DAKO (Carpinteria, CA, USA), respectively. Rabbit polyclonal antibodies for HER2 (A0485) were obtained from DAKO.

A Histofine Kit (Nichirei Bioscience, Tokyo, Japan), which employs the streptavidin-biotin amplification method was used in this study. Antigen retrieval was performed by heating the slides in an autoclave at 120°C for 5 min in antigen retrieval solution (pH 9.0; Nichirei Bioscience) for *BUB1* immunostaining or citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate (pH 6.0)) for other antibodies. Dilutions of primary antibodies used in this study were as follows: *BUB1*, 1/200; ER, 1/50; PR, 1/30; HER2, 1/200; Ki-67, 1/50; and γ-tubulin, 1/500. The antigen–antibody complex was visualized with 3,3'-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris–HCl buffer (pH 7.6), and

0.006% H_2O_2) and counterstained with hematoxylin. Human gastric carcinoma tissue was used as a positive control for *BUB1* antibody [16]. As negative controls of *BUB1* immunostaining, we used normal rabbit IgG instead of the primary antibody or no secondary antibody in this study.

Scoring of Immunoreactivity and Statistical Analysis

Immunoreactivity of *BUB1* was detected in the nucleus and/or cytoplasm of the breast carcinoma cells. Therefore, we separately evaluated *BUB1* immunoreactivity in the nucleus and cytoplasm, and the cases that had more than 10% of positive carcinoma cells were considered positive for nuclear and cytoplasmic *BUB1status*, respectively. Immunoreactivity for ER, PR, and Ki-67 was detected in the nucleus, and the immunoreactivity was evaluated in more than 1,000 carcinoma cells for each case, and their LI was subsequently determined. Cases with ER LI of more than 1% were considered ER-positive breast carcinoma in this study [17]. HER2 immunoreactivity was evaluated according to the grading system proposed in HercepTest (DAKO), and strongly circumscribed membrane-immunoreactivity of HER2 present in more than 10% carcinoma cells (score 3+) were considered positive. γ-Tubulin immunoreactivity was classified into three groups according to a previous report [20]. Briefly, percent of the positive cells in each case was scored 0 (less than 5%), 1 (5–25%), 2, (26–50%), 3 (51–75%), or 4 (more than 75%), as well as its immunointensity (0, negative; 1, weak; 2, moderate; and 3, intense). These scores were multiplied (range, 0–12) and then classified into the following three groups: low (the multiplied score 0–4), moderate (score 5–8), and high (score 9–12).

An association between signal intensity of the mitotic checkpoint genes and Ki-67 LI was evaluated using correlation coefficient (r) and regression equation. An association between *BUB1* immunohistochemical status and clinicopathological factors was evaluated by the Student's t test or a cross-table using the chi-square test. Disease-free and breast cancer-specific survival curves were generated according to the Kaplan–Meier method, and statistical significance was calculated using the log-rank test. Uni- and multivariate analyses were evaluated by a proportional hazard model (COX). P values of less than 0.05 were considered significant in this study. The statistical analyses were performed using the StatView 5.0J software (SAS Institute, Cary, NC, USA).

Results

Association Between Expression of Mitotic Checkpoint Genes and Proliferative Activity in the Breast Carcinoma Cases

Ki-67 antibody recognizes cells in all phases of the cell cycle except G0 (resting) phase [21], and Ki-67 LI is well-known to be closely correlated with the S phase fraction and mitotic index in the breast carcinoma [2]. When we examined an association between expression level of seven representative mitotic checkpoint genes evaluated by microarray and Ki-67 LI (Fig. 1), *BUB1* was positively associated with Ki-67 LI (P=0.0012, r=0.67) (Fig. 1a). Similar tendency was also detected in *BUB1B* (P=0.069) (Fig. 1b), *MAD2* (P=0.15) (Fig. 1e), *CDC20* (P=0.14) (Fig. 1f), and *TTK* (P=0.074) (Fig. 1g) and reverse tendency in *MAD1* (P=0.13) (Fig. 1d), but these did not reach statistical significance. The status of *BUB3* (Fig. 1c) was not associated with Ki-67 LI in this study. The microarray data of these genes were provided in Supplementary Table S1.

Associations of expression levels among these mitotic checkpoint genes were summarized in Table 1. Statistically significant positive associations were detected between *BUB1B* and *MAD2* (P=0.0002), CDC2 (P=0.0009), or *TTK* (P=0.0095), between *MAD2* and *CDC20* (P<0.0001) or *TTK* (P=0.0001), and between *CDC20* and *TTK* (P=0.0059). *BUB1* expression was not significantly associated with other genes examined.

BUB1 Immunolocalization in Human Breast Carcinoma Cases

Immunoreactivity of *BUB1* was detected in the nuclei and/or cytoplasm of breast carcinoma cells (Fig. 2a–c). *BUB1* immunoreactivity was also focally detected in the nuclei of epithelial cells in morphologically normal glands (Fig. 2d), while negative in the stroma. No significant *BUB1* immunoreactivity was detected in the negative control sections in this study (Fig. 2e).

Associations between nuclear *BUB1* immunohistochemical status and various clinicopathological parameters in breast carcinomas were summarized in Table 2. The number of *BUB1*-positive breast carcinomas was 42 out of 104 (40%) cases. Nuclear *BUB1* status was significantly associated with stage (P=0.0070), pathological tumor factor (pT) (P=0.023), lymph node metastasis (P=0.016), distant metastasis (P=0.041), histological grade (P=0.009), Ki-67 LI (P=0.003), and cytoplasmic *BUB1* status (P=0.0017), while no significant association was detected in patients' age, menopausal status, ER status, PR LI, and HER2 status.

Previous studies demonstrated that γ-tubulin immunoreactivity was closely associated with aberrations of centrosomes and/or chromosomes in the breast carcinoma [20, 22]. However, no significant association was detected between γ-tubulin immunoreactivity and nuclear *BUB1* status (P=0.46) in this study (Table 2).

The positive association between nuclear *BUB1* status and stage or distant metastasis was significant regardless of ER status of these cases, while significant association between nuclear *BUB1* status and pT, lymph node

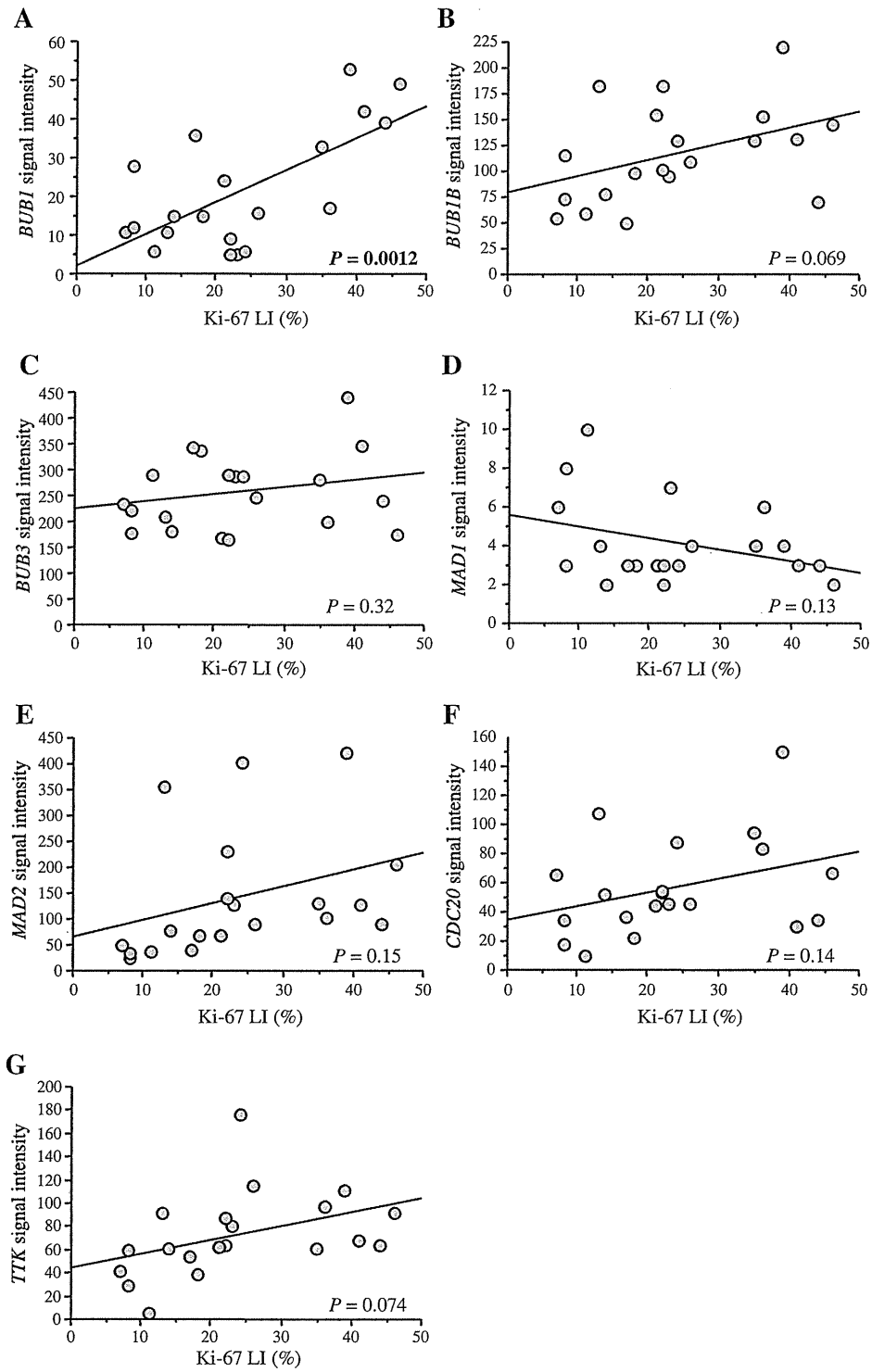

Fig. 1 Association between expression of mitotic checkpoint genes (i.e., *BUB1* (**a**), *BUB1B* (**b**), *BUB3* (**c**), *MAD1* (**d**), *MAD2* (**e**), *CDC20* (**f**), and *TTK* (**g**)) and Ki-67 LI in the breast carcinoma. Signal intensity of each gene was obtained from microarray, and Ki-67 LI was evaluated by immunohistochemistry. Statistical analysis was evaluated using correlation coefficient (*r*) and regression equation. *P* values less than 0.05 were considered significant and described as *boldface*

metastasis, histological grade, Ki-67 LI, or cytoplasmic *BUB1* status was detected only in ER-positive group (Table 3).

Cytoplasmic *BUB1* immunoreactivity was detected in 60 out of 104 (58%) breast carcinoma cases. Cytoplasmic *BUB1* status was marginally associated with Ki-67 LI in the breast carcinoma (P=0.052), but no significant association was detected between cytoplasmic *BUB1* status and clinicopathological parameters examined in this study (Table 4).

Table 1 Association among expression levels of seven mitotic checkpoint genes in 20 breast carcinomas

	BUB1B	*BUB3*	*MAD1*	*MAD2*	*CDC20*	*TTK*
BUB1	0.37	0.17	0.25	0.61	0.25	0.96
BUB1B		0.88	0.19	**0.0002**	**0.0009**	**0.0095**
				(r=0.74)	(r=0.68)	(r=0.57)
BUB3			0.99	0.25	0.36	0.74
MAD1				0.26	0.53	0.082
MAD2					**<0.0001**	**0.0001**
					(r=0.80)	(r=0.76)
CDC20						**0.0059**
						(r=0.59)

Data are presented as P values. P values less than 0.05 were considered significant and described as boldface

Association Between *BUB1* Status and Clinical Outcome of the Patients

In order to examine an association between *BUB1* status and prognosis of the patients precisely, we excluded stage IV cases and used stages I to III breast carcinoma patients (n=91) in the following analyses. Nuclear *BUB1* status was significantly associated with an increased incidence of recurrence (P=0.0001) as demonstrated in Fig. 3a, whereas cytoplasmic *BUB1* status was not (P=0.59) (Fig. 3b). The multivariate analysis revealed that lymph node metastasis (P=0.0022) and nuclear *BUB1* status (P=0.0056) were independent prognostic factors for disease-free survival with relative risks over 1.0 (Table 5).

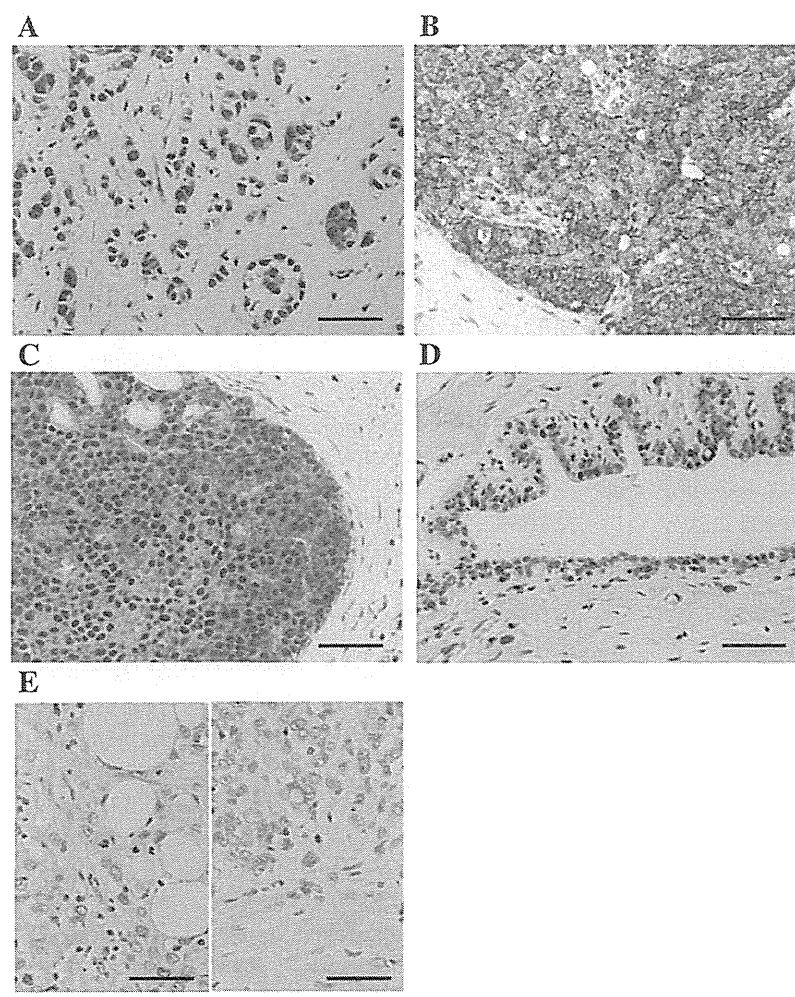

Fig. 2 Immunohistochemistry for *BUB1* in the breast carcinoma. *BUB1* immunoreactivity was detected in the nucleus (**a**), cytoplasm (**b**), or both nucleus and cytoplasm (**c**) of the carcinoma cells. *BUB1* immunoreactivity was focally detected in the nucleus of morphologically normal mammary epithelium (**d**). **e** Negative control sections of *BUB1* immunohistochemistry (*left panel*: normal rabbit IgG used instead of the primary antibody and *right panel*: no secondary antibody). *Bar*=100 mm, respectively

Table 2 Association between nuclear *BUB1* immunohistochemical status and clinicopathological parameters in 104 breast carcinomas

	Nuclear *BUB1* status		*P* value
	+ (n=42)	− (n=62)	
Patient age[a] (years)	54.2±1.6	56.0±1.5	0.44
Menopausal status			
Premenopausal	17 (16%)	21 (20%)	0.49
Postmenopausal	25 (24%)	41 (39%)	
Stage			
I	6 (6%)	23 (22%)	**0.0070**
II	19 (18%)	28 (27%)	
III	7 (7%)	8 (7%)	
IV	10 (10%)	3 (3%)	
Pathological tumor factor (pT)			
pT1	11(11%)	30 (29%)	**0.023**
pT2-4	31 (30%)	32 (31%)	
Lymph node metastasis			
Positive	25 (24%)	22 (21%)	**0.016**
Negative	17 (16%)	40 (38%)	
Distant metastasis			
Positive	10 (10%)	3 (3%)	**0.041**
Negative	32 (31%)	59 (57%)	
Histological grade			
1 (well)	1 (1%)	19 (18%)	**0.009**
2 (moderate)	21 (20%)	27 (26%)	
3 (poor)	20 (19%)	16 (15%)	
ER status			
Positive	32 (31%)	50 (48%)	0.58
Negative	10 (10%)	12 (12%)	
PR LI[a] (%)	28.0±3.7	21.5±4.6	0.27
HER2 status			
Positive	14 (14%)	12 (12%)	0.13
Negative	28 (27%)	50 (48%)	
Ki-67 LI[a] (%)	26.8±2.7	14.6±1.9	**0.0003**
Cytoplasmic *BUB1* status			
Positive	32 (31%)	28 (27%)	**0.0017**
Negative	10 (10%)	34 (33%)	
γ-Tubulin immunoreactivity			
Low	16 (15%)	18 (17%)	0.46
Moderate	15 (14%)	21 (20%)	
High	11 (11%)	23 (22%)	

P values less than 0.05 were considered significant and described as boldface

[a] Data are presented as mean±SEM. All other values represent the number of cases and percentage

Breast cancer-specific survival curves of *BUB1* status were summarized in Fig. 3c and d. A significantly positive correlation (P=0.0007) was detected between nuclear *BUB1* status and adverse clinical outcome of the patients examined, but cytoplasmic *BUB1* status was not associated (P=0.72). In the univariate analysis (Table 6), nuclear *BUB1* status (P=0.011), histological grade (P=0.018), Ki-67 LI (P=0.026), and lymph node metastasis (P=0.043) were all detected as significant prognostic variables for breast cancer-specific survival in this study. However, a following multivariate analysis revealed that only nuclear *BUB1* status was independent prognostic factor with a relative risk over 1.0 (P=0.043), whereas histological grade (P=0.21), Ki-67 LI (P=0.75), and lymph node metastasis (P=0.087) were all not significant.

In our present study, 51 patients received tamoxifen therapy following surgery as an adjuvant treatment in ER-positive stages I-III breast carcinoma cases, and nuclear *BUB1* status was significantly associated with an increased risk of recurrence in these patients (P=0.0079) (Fig. 4a). Similar tendency was detected between nuclear *BUB1* status and breast cancer-specific survival of the patients, although *P* value did not reach statistical significance (P=0.14). Significant association between nuclear *BUB1* status and clinical outcome of the patients was also detected in 67 patients who received adjuvant chemotherapy (P=0.0001 for disease-free survival (Fig. 4b) and P=0.0028 for breast cancer-specific survival). Nuclear *BUB1* status was significantly associated with an increased risk of recurrence (Fig. 4c) and worse prognosis in the ER-negative stages I-III cases (n=19), although *P* values were not available because no patient had recurrent disease or died in the group of these nuclear *BUB1*-negative cases.

Discussion

Results of our present study demonstrated that *BUB1* expression level was significantly associated with Ki-67 LI in the breast carcinoma cells, and similar tendency was also detected in *BUB1B*, *MAD2*, *CDC20*, and *TTK*. Yuan et al. [14] previously reported that mRNA levels of mitotic checkpoint genes, such as *BUB1*, *BUB1B*, *BUB3*, *MAD1*, *MAD2*, *CDC20*, and *TTK*, were almost uniformly increased in breast carcinoma cell lines compared with MCF10A and mammary epithelial cells. Overexpression of *BUB1*, *BUB1B*, *BUB3* [23, 24], and *MAD2* [25] was also reported in the gastric carcinoma cells. In particular, Grabsch et al. [24] did report a positive association between *BUB1*, *BUB1B*, or *BUB3* and Ki-67 mRNA levels in the gastric carcinoma. Association between *BUB1* mRNA level and Ki-67 LI was also reported in the salivary gland tumors [15]. Results of these studies above are all consistent with those of our present study. However, *MAD1* expression tended to be inversely associated with Ki-67 LI in our present study. Han et al. [26] reported that MAD1 expression was significantly reduced in poorly differentiated breast carcinomas, which may partly explain our present finding. These results