of estrogen for breast cancers can therefore be through the circulation (endocrine), from within the breast stroma (paracrine) or through synthesis by the tumor cell (autocrine) [1, 2]. Intra-tumoral estrogen production has been directly demonstrated by measuring the conversion of radio-labeled androgen to estrogen in breast cancer biopsy material [1–3]. However correlations between biochemical measurements of intra-tumoral estrogen synthesis and clinical outcomes have not been firmly established, largely because in vivo assays of aromatase activity are difficult to execute in a large numbers of patients [4–6]. As alternative approaches, aromatase immunohistochemistry (IHC) and measurements of aromatase mRNA levels have been explored [7, 8]. However, most investigators have not validated their IHC assays against the "gold standard" of a biochemical assay for intra-tumoral aromatase activity. Our group has recently developed and characterized a monoclonal antibody against aromatase. The antibody has been utilized in IHC studies which demonstrated positive correlations between aromatase IHC scores and intra-tumoral aromatase activity [9] and aromatase mRNA expression measurements [10] in breast cancer specimens.

In this investigation we applied the aromatase IHC assay to formalin-fixed paraffin-embedded biopsy samples accrued from patients enrolled onto the P024 neoadjuvant endocrine therapy study, a Phase III double blind randomized trial that compared four months neoadjuvant tamoxifen with an equivalent period of letrozole treatment [11–13]. The design of this study provided a valuable opportunity to evaluate simultaneously the relationship between tumor aromatase expression and response to neoadjuvant endocrine therapy as well as the long-term outcomes for patients receiving adjuvant tamoxifen treatment.

Methods

Study population and tumor bank

The P024 protocol compared four months neoadjuvant letrozole with tamoxifen in post-menopausal women with clinical stage II and III hormone receptor positive (classified as at least 10% nuclear staining for ER and/or PgR) breast cancers that were ineligible for breast conservative surgery [11]. The tumor bank characteristics, ER and Ki67 measurements have been described previously [12, 13]. Tumor grade, tumor histological subtype, pathological staging information and long-term outcomes were collated from case report forms. The long-term outcomes and the development of the preoperative endocrine prognostic index (PEPI) based on pathological stage, and the ER status and Ki67 expression level of the surgical specimen has also been published [14].

Aromatase immunohistochemistry

The aromatase monoclonal antibody #677 was raised against native recombinant human aromatase protein. Details of its characterization and utilization for IHC have been previously reported [9]. Tissue sections were immunostained by a biotin-streptavidin method using a Histofine kit (Nichirei Co. Ltd., Tokyo, Japan). The antigen-antibody complex was visualized with 3.3′-diaminobenzidine solution (DAB) and counterstained with hematoxylin. Evaluation of aromatase IHC was performed by assessing the approximate percentage of cells staining (proportion score) and classifying the level into four groups: $0 = <1\%$, $1 = 1–25\%$, $2 = 26–50\%$, and $3 = >50\%$ immuno-positive cells. The relative intensity of aromatase immune-positive cells was classified as follows: 0 = no immunoreactivity, 1 = weak, 2 = moderate and 3 = intense immunoreactivity. When aromatase immunoreactivity was evaluated as a semi-continuous variable, a total score was applied that was composed of the proportion score + relative immuno-intensity score (SIP score). For contingency table analysis, aromatase staining was classified as any staining present versus absent staining. Immunohistochemical staining patterns of normal ducts, stromal cells, adipose cells and carcinoma cells were evaluated separately.

Statistics

All P values reported were two sided; $P \leq 0.05$ were considered to be statistically significant. There was no adjustment for multiple testing. The median and interquartile range of the aromatase SIP score was calculated to show the distribution of scores. Kendall's rank correlation coefficients were used to assess relationship between aromatase SIP values and Ki67, ER, and tumor size since aromatase SIP values were ordinal variables and not normal distributed. Fisher's exact and Chi squared tests were used to define associations between aromatase expression status and clinical and cell cycle responses. The non-parametric Mann–Whitney test was applied to compare differences in Ki67 changes between aromatase expression positive and aromatase expression negative tumors. The 95% confidence interval of the geometric Ki67 mean was calculated to show the size of effects in pair-wise comparisons. Relapse-free survival (RFS) was defined as the interval between randomization and the earliest subsequent breast cancer event (all local or systemic recurrences, there were no new breast primaries recorded in this data set). Breast cancer-specific survival (BCSS) was defined as the

interval between randomization and the date of death after breast cancer relapse. For univariable analysis, survival curves were estimated by the Kaplan–Meier product-limit method, with a two-sided log-rank to assess statistically significant differences. We subsequently applied a multivariate Cox proportional hazards regression model to evaluate the independent prognostic relevance of aromatase expression within the context of other independently prognostic variables that were obtained upon analysis of the surgical specimen obtained after completion of neoadjuvant endocrine therapy: i.e. pathological tumor size, lymph node status, ER and Ki67 levels [14]. The REMARK analysis for the multivariable analysis has also been reported [14]. All statistical analyses were performed using SAS 9.1.2 (SAS Institute Inc., Cary NC USA).

Results

Aromatase expression and correlation with baseline pathological and clinical variables

Initially four cellular components were scored for aromatase expression (fibroblast cells, adipose cells, benign breast duct cells and invasive cancer cells). However benign ducts and adipose tissue were very inconsistently present in the slides available. Thus, only stromal cell scores and invasive cancer cell scores could be adequately studied in terms of correlations with clinical parameters. Ultimately aromatase analysis was conducted on 197 cases in which central analysis confirmed ER+ status and 23 cases in which the ER status was known to be ER negative in the central laboratory (with a cut point of Allred score of 0 or 2 as the definition of negative). Of these 197 ER+ cases, 192 (96 on letrozole, 96 on tamoxifen) had sufficient tumor cells on specimens to qualify for the analysis presented in this report. Aromatase expression SIP score in the stomal cell and tumor cell compartments were highly correlated (Kendall's Tau 0.46, $P = 0.0001$, Fig. 1a) Tumor cell aromatase SIP score was positively correlated with ER levels as a continuous score (Kendall's Tau $P = 0.006$, Fig. 1b), however there was no significant correlation with progesterone receptor (PgR) level (data not shown). Finally the aromatase SIP score in the cancer compartment was inversely associated with Ki67 level (Kendall's Tau $P = 0.003$ Fig. 1c). To examine correlations between aromatase expression and dichotomized clinical variables the aromatase staining score was reduced to simple present or absent categories. Of the variables examined, both stroma and tumor epithelial aromatase expression were associated with smaller clinical tumor size at baseline and ER positive status as a dichotomous variable (Allred 0–2 vs. Allred 3–8) but aromatase status

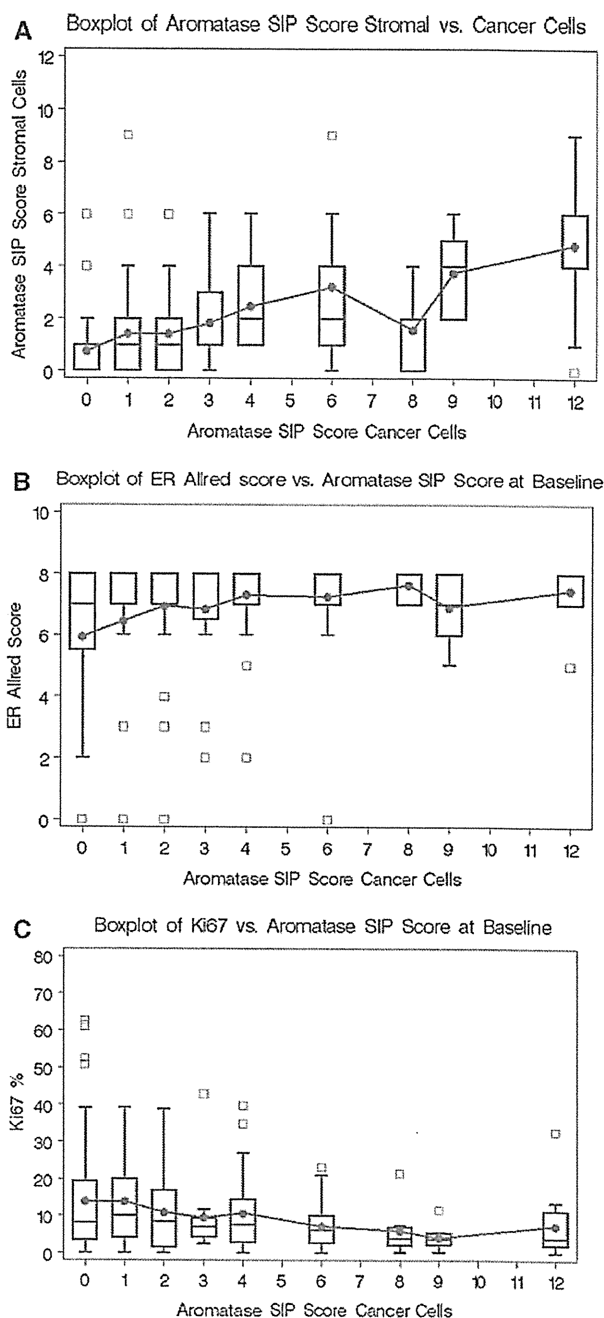

Fig. 1 Correlations between the site of aromatase expression, ER and Ki67 as semi-continuous variables. Box plots comparing the distributions of aromatase SIP scores in stromal cells and cancer cells (**a**), aromatase SIP scores in cancer cells and ER Allred scores (**b**) and Ki67 percentage and aromatase SIP scores in cancer cells (**c**) at baseline. The large boxes stretch from the 25th to 75th percentile, the lines crossing the boxes are medians, the dots are means and the small boxes are outliers.

(present vs. absent) did not interact with the other factors examined (patient age, tumor grade, lymph node status, PgR and HER2 status) (Table 1).

Table 1 Patients and tumors characteristics by location of aromatase protein expression status at baseline

Characteristics	Aromatase protein expression [n (%)][b]			
	Tumor epithelial aromatase		Stromal aromatase	
	Negative	Positive	Negative	Positive
Treatment				
Tamoxifen	24 (53%)	72 (49%)	29 (54)	63 (48)
Letrozole	21 (47%)	75 (51%)	25 (46)	69 (52)
P-value	0.6102		0.4605	
Age (year)	66.8	67.6	67.6	67
P-value[c]	0.6214		0.6969	
Clinical tumor size (cm)	5.7	4.8	5.5	4.7
P-value[c]	0.0144		0.0398	
Pre treatment grade				
I	4 (10%)	16 (13%)	4 (8%)	16 (15%)
II/III	38 (90%)	103 (87%)	47 (92%)	90 (85%)
P-value	0.5971		0.3064	
Pathological tumor size				
≤20 mm	11 (27%)	44 (32%)	11 (27%)	44 (32%)
>20 mm	30 (73%)	95 (68%)	30 (73%)	95 (68%)
P-value	0.5567		0.5567	
Pathological node status				
Negative	16 (41%)	55 (43%)	21 (47%)	50 (43%)
Positive	23 (59%)	72 (57%)	24 (53%)	65 (57%)
P-value	0.8017		0.7160	
HER2 status[a]				
Negative	40 (91%)	140 (95%)	50 (93%)	125 (95%)
Positive	4 (9%)	7 (5%)	4 (7%)	6 (5%)
P-value	0.2806		0.4810	
ER status[c]				
Negative	11 (20%)	12 (8%)	14 (20%)	9 (6%)
Positive	44 (80%)	148 (93%)	55 (80%)	131 (94%)
P-value	0.0098		0.0027	
PgR status[c]				
Negative	16 (36%)	48 (33%)	20 (37%)	43 (33%)
Positive	28 (64%)	98 (67%)	34 (63%)	87 (67%)
P-value	0.6688		0.6072	

[a] HER2 IHC with fluorescence in situ hybridization confirmation and IHC for ER and PgR were performed as previously described [12]

[b] Aromatase protein expression considered positive if any aromatase IHC staining was present

[c] For age and clinical tumor size the student's t test was used to compare the aromatase positive and negative groups. For binary variables the X^2 test was applied with Fisher's exact test if a count in any cell was less than 5

Aromatase expression and clinical or radiological response to neoadjuvant letrozole or tamoxifen

A series of contingency tables were examined to identify interactions between aromatase expression status and response (Table 2). In the P024 study, response was recorded according to clinical measurements, ultrasound and mammography. There was no evidence of interactions with any of the response definitions, whether the stroma or the tumor cell aromatase status was examined as the interacting factor or whether letrozole or tamoxifen treated cases were considered separately. Consistent with a lack of an influence on endocrine therapy responsiveness, there was no interaction with treatment-induced changes in Ki67 or absolute post-treatment Ki67 levels in either tamoxifen or letrozole-treated tumor samples (Table 3).

Aromatase expression and relapse-free survival and breast cancer-specific survival

Although there was no association with neoadjuvant response or Ki67 changes, the baseline interactions between aromatase expression, higher ER levels and lower Ki67 levels suggested the possibility that aromatase expression could be a favorable prognostic biomarker for patients undergoing adjuvant endocrine therapy. We therefore examined the impact of aromatase expression on RFS and BCSS (Fig. 2). Tumor aromatase expression was

Table 2 Analysis of clinical, mammogram and ultrasound response data according to aromatase protein expression status in tamoxifen or letrozole treated patients

Responses	Aromatase protein expression [*n* (%)]			
	Tumor epithelial aromatase		Stromal aromatase	
	Negative	Positive	Negative	Positive
Clinical response[a]				
Letrozole only				
No	6 (29%)	24 (32%)	11 (44%)	18(26)
Yes	15 (71%)	51 (68%)	14 (56%)	51(74)
P-value	0.7657		0.0984	
Tamoxifen only				
No	12 (50%)	37 (51%)	13 (45%)	34 (54%)
Yes	12 (50%)	35 (49%)	16 (55%)	29 (46%)
P-value	0.9067		0.4177	
Fused				
No	18 (40%)	61 (41%)	24 (44%)	52 (39%)
Yes	27 (60%)	86 (59%)	30 (56%)	80 (61%)
P-value	0.8587		0.5259	
Mammo response[a]				
Letrozole only				
No	13 (62%)	47 (63%)	17 (68%)	42 (61%)
Yes	8 (38%)	28 (37%)	8 (32%)	27 (39%)
P-value	0.9494		0.5297	
Tamoxifen only				
No	19 (79%)	54 (75%)	23 (79%)	48 (76%)
Yes	5 (21%)	18 (25%)	6 (21%)	15 (24%)
P-value	0.6803		0.7418	
Fused				
No	32 (71%)	101 (69%)	40 (74%)	90 (68%)
Yes	13 (29%)	46 (31%)	14 (26%)	42 (32%)
P-value	0.7604		0.4278	
Ultrasound response[a]				
Letrozole only				
No	12 (67%)	39 (56%)	16 (73%)	34 (52%)
Yes	6 (33%)	31 (44%)	6 (27%)	31 (48%)
P-value	0.4038		0.0959	
Tamoxifen only				
No	13 (68%)	44 (64%)	15 (60%)	41 (69%)
Yes	6 (32%)	25 (36%)	10 (60%)	18 (31%)
P-value	0.7085		0.4016	
Fused				
No	25 (68%)	83 (60%)	31 (66%)	75 (60%)
Yes	12 (32%)	56 (40%)	16 (34%)	49 (40%)
P-value	0.3845		0.5116	

Response rate refers to the percentage of patients with a complete or partial response

[a] Response definitions by WHO criteria have been previously reported [11]. The X^2 test was applied with Fisher's exact test if a count in any cell was less than 5

confirmed to have a modest association with a more favorable disease course, with fewer relapse events over time and a significant univariable log rank test $P = 0.04$ (Fig. 2a) and more prolonged breast cancer survival (Fig. 2b $P = 0.01$). To determine the independence of baseline aromatase expression as a prognostic marker in our established multivariable models based on the post-treatment surgical sample, the baseline aromatase status was analyzed in the context of the preoperative endocrine relapse index (PEPI) (Table 4) [14]. In the PEPI model pathologic tumor size (T1/2 vs. T3/4), pathological nodal status (negative vs. positive), Ki67 per natural log interval and ER status post therapy (Allred 0–2 vs. Allred 3–8) have been found to be independent factors for RFS and BCSS [14]. When tumor aromatase status was entered into a multivariable Cox model containing these four factors, the presence of aromatase expression in the baseline specimen behaved as an independent favorable prognostic biomarker for both RFS ($P = 0.01$, HR 2.3 95% 1.2–4.6 for absent expression) (Table 4A) and BCSS ($P = 0.008$, HR 3.76 95% CI 1.4–10.0 for absent expression) (Table 4B).

Discussion

The clinical significance of intra-tumoral estrogen production has been debated ever since the phenomenon was first documented by Miller et al., in 1974 [15] through the detection of the conversion of radio-labeled androgen to estradiol within breast cancers in vitro. This potential exists in about 60–70% of breast cancers [1–3]. Subsequently infusion studies with radioactive androgens showed that estrogen biosynthesis occurred in situ within the breast [16, 17] and the presence of mRNA for aromatase, the key enzyme in estrogen production, was also demonstrated in breast cancers and adipose tissue [8]. Because aromatase is the last step in the biosynthetic pathway for estradiol, the enzyme has become a critical target for pharmacological inhibitors that achieve endocrine deprivation for post-menopausal patients requiring endocrine treatment for ER+ breast cancer. Consequently third-generation aromatase inhibitors have evolved as the new standard of care for breast cancer treatment for all stages of the disease. It was therefore logical to address the possibility that the presence of aromatase within breast cancers is associated with a particular requirement for estrogen for growth and therefore whether aromatase expressing tumors are more likely to respond to endocrine therapy in general, and to aromatase inhibitors in particular.

The number of studies examining these relationships is few, have utilized small numbers of tumors and come to limited (often conflicting) conclusions [4, 18, 19]. The

Table 3 Paired Ki67 data before and after letrozole/tamoxifen therapy according to aromatase protein expression status in breast cancer cells/ stromal cells

Ki67 [Geometric mean (95% CI)][a]	Aromatase protein expression			
	Cancer cells		Stromal cells	
	Negative	Positive	Negative	Positive
Letrozole only				
Pre	5.54 (2.54–12.08)	3.56 (2.47–5.14)	3.64 (1.70–7.82)	3.92 (2.69–5.72)
Post	0.70 (0.33–1.49)	0.49 (0.31–0.75)	0.88 (0.38–2.04)	0.44 (0.29–0.68)
P-value[b]	0.0037	0.0001	0.0083	0.0001
Tamoxifen only				
Pre	5.97 (3.17–11.24)	5.63 (4.18–7.58)	7.75 (4.46–13.47)	4.67 (3.39–6.44)
Post	1.72 (0.75–3.97)	1.36 (0.88–2.09)	1.61 (0.76–3.39)	1.23 (0.77–1.95)
P-value[b]	0.0117	0.0001	0.0007	0.0001

[a] 95% CI: confidence Interval

[b] Wilcoxon signed rank test was used to compare paired Ki67 data within each group defined by aromatase expression status

major reason for this is that the low abundance of aromatase in the breast requires sophisticated, time-consuming and labor intensive methodology and relatively large amounts of fresh tissue. This has precluded routine use in large clinical trials. However the availability of an antibody which can specifically detect aromatase in fixed archival breast cancers has changed this. We can now report results on the presence (and semi-quantitative levels) of aromatase in tumor material obtained from a randomized trial of neoadjuvant endocrine therapy (P024).

Before discussing the findings it is worth considering methodological issues and potential limitations of the study. Firstly, IHC estimation of protein provides no information on activity and protein may be present that is deactivated or inhibited [20]. This certainly will be the case in patients treated with aromatase inhibitors. For this reason we have excluded outcome correlations with aromatase status in "on treatment" samples because we have not validated relationships between aromatase activity and expression in the presence of an endocrine agent. Secondly, because aromatase is present in different compartments of the breast (and at different levels) complete assessment requires quantification of multiple tissue types and an estimate of the relative amounts of each compartment. In this study, to simplify these confounders, we have not used assessments in adipose and benign tissue of tissue sections which were generally low in staining score and proportion. Aromatase scores were highest in the malignant and stromal compartments of breast cancers. However, these were highly related in breast cancers suggesting a field effect of trophic factors regulating aromatase. We have therefore restricted our correlations to the status of the cancer cells which were reliably present in all the samples eligible for analysis and therefore more consistent to score.

In terms of demographics we have combined the two arms of the P024 trial for long term outcome analysis, not discriminating between patients subsequently treated with tamoxifen or letrozole since all patients received tamoxifen as adjuvant therapy. This has formed a database which represents the largest published series of breast cancers assessed by aromatase IHC. The results show that tumor aromatase was positively and significantly related to smaller tumor size and ER level/status. These findings would be consistent with data published by members of the group on aromatase activity [4] but not with others using IHC with a different antibody [7]. A significant inverse correlation was observed with the proliferation marker, Ki67. To the best of our knowledge there have been no other published studies relating tumor aromatase to proliferation.

In terms of endocrine responsiveness, no significant association was detected between tumor aromatase and clinical response to either letrozole or tamoxifen. While positive correlations have been reported between the presence of in vitro and in vivo aromatase activity and response to aromatase inhibitors, these relationships were not strong and were observed in advanced disease, not in the neoadjuvant setting [18, 19] Other studies on response to tamoxifen have been negative. Thus, the response to endocrine therapy does not appear to be strongly modulated by whether the source of estrogen is autocrine or endocrine.

Despite a failure to observe significant relationships between aromatase expression and clinical or biomarker response to treatment in the neoadjuvant phase of the study, significant associations were found between the presence of tumor aromatase expression and long-term outcome following neoadjuvant treatment. Thus, tumors with positive aromatase scores had significantly greater

Table 4 Univariate and multivariate analysis of pathological tumor size, node status, post-treatment Ki67, post-treatment ER and pre treatment aromatase status[a]

A

Factor definitions	No. of patients in each group	No. of events/ No. of patients	Relapse-free survival			
			Univariable analysis		Multivariate analysis	
			HR (95% CI)	*P*	HR (95% CI)	*P*
Tumor size[a] (T1/2 vs. T3/4)	138/33	47/171	2.7 (1.4–5.0)	0.002	2.82 (1.36–5.85)	0.006
Node status (Yes vs. No)	90/69	44/159	3.9 (1.8–8.4)	0.0005	3.44 (1.58–7.48)	0.002
Ki67 level, per 2.7 fold increase[b]		48/174	1.4 (1.2–1.6)	0.0002	1.1 (1.02–1.09)	0.003
ER Allred[c] (0.2 vs. 3–8)	16/157	48/173	2.4 (1.0–5.3)	0.04	2.74 (1.1–6.67)	0.03
Aromatase status[d] (not present versus present)	37/132	48/169	1.88 (1.01–3.47)	0.04	2.34 (1.2–4.58)	0.01

B

Factor definitions	No. of patients in each group	No. of events/ No. of patients	Breast cancer-specific survival			
			Univariable analysis		Multivariate analysis	
			HR (95% CI)	*P*	HR (95% CI)	*P*
Tumor size (T1/2 vs. T3/4)	138/33	24/171	3.5 (1.5–8.3)	0.004	3.42 (1.21–9.66)	0.02
Node status (Yes vs. No)	90/69	22/159	4.6 (1.4–15.8)	0.01	4.05 (1.14–14.38)	0.03
Ki67 level, per 2.7 fold increase		25/174	1.4 (1.1–1.7)	0.009	1.05 (1.0–1.11)	0.06
ER Allred (0.2 vs. 3–8)	16/157	25/173	4.3 (1.6–11.7)	0.005	7.98 (2.58–24.7)	0.0003
Aromatase status (not present versus present)	37/132	24/169	2.82 (1.2–6.63)	0.02	3.76 (1.42–9.98)	0.008

[a] The four elements of the preoperative endocrine relapse index (PEPI) score (pathological T and N stage, surgical specimen ER and Ki67 status has been previously described [14]

[b] Surgical specimen Ki67 was analyzed as the natural log interval, or per 2.7 fold increase according to the original scale of percentage values [14]

[c] The ER analysis refers to the post-treatment values, before treatment all the tumors in this data set were ER positive. In the PEPI model, an Allred cut off of 0 or 2 is used to define ER negative

[d] The aromatase expression status was defined as present or positive if any positive staining presented in invasive breast cancer cells. Table 4A and B shows the RFS data and BCSS data, respectively

RFS and BCSS. It is not possible to ascertain whether this is directly caused by increased sensitivity to endocrine therapy in the adjuvant setting. However, the lack of association of response in the neoadjuvant situation would not be compatible with this. Furthermore the positive correlations with small clinical size and ER status levels (favorable prognostic biomarkers) and the inverse correlation with Ki67 (a poor prognosis biomarker) suggest that aromatase positive tumors may be inherently less aggressive. This is supported by data from multivariable analyses in which tumor aromatase scores predicted for long-term outcome independently of other factors that have been shown to be predictive for outcome in the post neoadjuvant endocrine therapy setting. This finding also implies that the most accurate models for the prediction of outcomes for patients with ER+ disease may combine baseline prognostic biomarker analysis, in combination with the "on-treatment" predictive biomarker analysis derived from an analysis of the tumor after several months of endocrine treatment [14].

It is therefore suggested that routine IHC measurements of aromatase in breast cancer will not generally aid

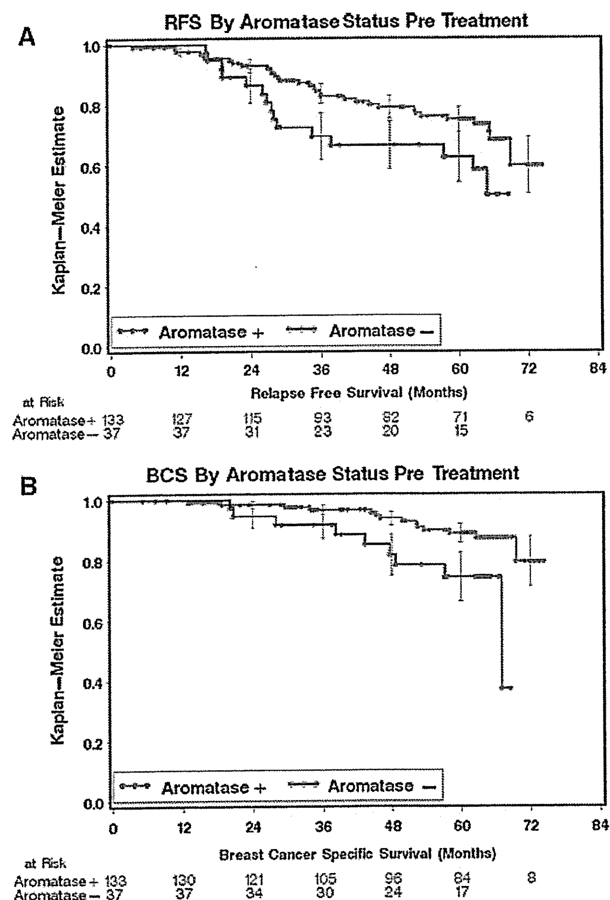

Fig. 2 Kaplan–Meier curves for relapse-free and breast cancer-specific survival by aromatase protein expression status in cancer cells. (**a**) Relapse-free survival (RFS) for patients with aromatase protein expression positive (green or upper curve) versus negative (red or lower curve) in cancer cells; (**b**) Breast cancer-specific survival (BCSS) for patients with aromatase protein expression positive (green or upper curve) versus negative (red or lower curve) in cancer cells; Censorship observations are marked with open circles; log rank tests were used to estimate the difference between Kaplan–Meier curves for RFS and BCSS. 95% confidence intervals are provided on each curve

prediction of neoadjuvant response to endocrine therapy, but may help identify ER positive tumors with favorable long-term outcomes.

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Runx2 in human breast carcinoma: its potential roles in cancer progression

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Runx2 has been proposed as one of the pivotal factors in the process of osteogenesis and metastasis in human malignancies including breast cancer, but its details have not been evaluated. Therefore, in this study, we evaluated its expression in human breast cancer using immunohistochemistry. One hundred and thirty-seven formalin-fixed and paraffin-embedded breast cancer specimens were used in this analysis of immunohistochemical study. Immunoreactivity was evaluated using the labeling index (LI). Runx2 immunoreactivity was detected in both carcinoma and stromal cells, as well as non-pathological ductal cells. The nuclear LI of Runx2 in carcinoma cells was associated with the clinical stage, histological grade and HER2 status of the patients examined. In addition, among the patients not associated with distant metastasis, those with high Runx2 LI demonstrated a significantly worse clinical outcome than those with a low LI. This was more pronounced in the group of estrogen receptor (ER)-negative cases. In addition, both univariate and multivariate analyses demonstrated that the Runx2 LI in breast carcinoma cells turned out an independent prognostic factor. Results of our present study demonstrated that Runx2 plays very important roles in the progression of breast cancer, especially in those of ER-negative cases. (*Cancer Sci* 2010; 101: 2670–2675)

Breast cancer is one of the most common malignancies in women worldwide. Recently, the potential association of breast cancer with its bone metastasis has been evaluated from different perspectives and, in particular, the process of osteolysis itself in its metastatic sites has been proposed to facilitate breast cancer progression.[1] It is also well known that breast carcinoma cells themselves secrete parathyroid-hormone-related peptide (PTHrP), which stimulates osteoblasts in the microenvironment of bone metastatis.[2] Osteoblasts at the sites of metastasis are also considered to secrete a receptor activator of NFκB ligand (RANKL) to facilitate the process of transition from mesenchymal cells into functional osteoclasts, which subsequently resorb bone.[3–7] In normal human adult skeleton, bone is constantly renewed or maintained through the coordinated activities of both osteoclasts and osteoblasts.[8] Metastatic breast carcinoma cells are seeded into the bone microenvironment, which results in the maturation of osteoclasts.[9] These subsequently formed osteolytic foci are associated with bone resorption, which eventually leads to the release of growth factors including transforming growth factor- β (TGF-β) and several insulin-like growth factors (IGF) from the collapsed bone matrix.[10,11] These factors are considered to subsequently mediate tumor cell proliferation at the sites of bone metastasis.

The Runt-related transcription factors 1-3 (Runxs1-3) have been shown to be required for the process of organogenesis, and mutations in these genes have been reported to be linked to several types of cancer development.[12] For instance, Runx1 and Runx3 mutations were reported to promote leukemia[13,14] and gastric cancers,[15] respectively. Among these Runx families, Runx2 plays a pivotal role in the process of bone formation or osteogenesis[16–19] and deregulation of Runx2 itself is associated with the development of osteosarcoma.[20,21] Runx2 was also reported to be highly expressed in both prostate and breast carcinoma cell lines, which can metastasize to bone in various transplanted models.[22–24] Loss of function of the Runx2 gene in the mouse was also reported to result in increased cell proliferation of *ex vivo* skeletal lineage cells.[25,26] Expression of Runx2 was also reported in mammary epithelial cells of the mouse.[27,28] In addition, aberrant Runx2 expression has been reported in breast and prostate primary tumors.[22,25] Runx2 was reported to be involved in the regulation of a mammary-gland-specific β-casein gene and osteopontin.[22,28,29] In regard to its potential roles at the sites of breast carcinoma metastasis to the bone, Runx2 was reported to regulate PTHrP expression of metastatic breast carcinoma cells in the microenvironment of bone metastasis and the cell cycle of carcinoma cells themselves.[30] Runx2 was also shown to modulate several factors, which can contribute to facilitating the process of metastasis including vascular endothelial growth factor (VEGF),[31] several matrix metalloproteinases (MMP)[24,32] and bone sialoprotein.[33] However, to the best of our knowledge, its roles in the early stage of breast cancer patients have not been studied at all. In addition, the correlation of Runx2 nuclear immunoreactivity in breast carcinoma cells and histopathological features of breast cancer were reported,[34] but the correlation between Runx2 expression and prognosis has still remained unknown.

Among the anti-estrogen therapies available in cases with estrogen receptor (ER)-positive breast carcinoma, the administration of selective estrogen receptor modulator (SERM) or aromatase inhibitor (AI) has been considered the gold standard.[35,36] However, it is well known that ovarian suppression and administration of AI frequently results in osteoporosis.[36–39] The suppression of estrogenic actions in osteoclasts results in inhibition of their apoptosis and enhancement of their maturation.[36–39] Therefore, both suppression of estrogenic actions and elevated Runx2 expression in metastatic breast carcinoma cells might enhance the development of osteoporosis in these patients.

Therefore, in the present study, we evaluated the status of nuclear Runx2 immunoreactivity in breast carcinoma cells and correlated the findings with stage, histological grade, ER status and HER2 expression of the patients in order to study its clinicopathological significance.

Materials and Methods

Breast carcinoma cases. One hundred and thirty-seven cases of invasive ductal carcinoma of the breast were retrieved from

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the surgical pathology files of the Department of Pathology, Tohoku University Hospital, Sendai, Japan. Breast tissue specimens were obtained from Japanese female patients who underwent a mastectomy during 1988–1999 in the Department of Surgery, Tohoku University Hospital, Sendai city, Japan. The mean age was 52.9 years (range, 22–81 years). None of the patients examined in the present study received chemotherapy, administration of trastuzumab or irradiation prior to surgery. The mean follow-up time was 81 months (range, 1–151 months). All of the specimens had been fixed in 10% formalin at room temperature and embedded in paraffin wax. Research protocols for this study were approved by the Ethics Committee at Tohoku University School of Medicine (approval number 2005-178).

Antibodies. Mouse monoclonal antibody for human Runx2 was purchased from Abnova Corporation (Taipei, Taiwan). The characterization of this antibody has been previously reported using both immunoblotting and immunohistochemistry.[40] Monoclonal antibodies for estrogen receptor α (ER1D5), progesterone receptor (PR; MAB429) and Ki-67 (MIB1) were purchased from Immunotech (Marseille, France), Chemicon (Temecula, CA, USA) and DAKO (Carpinteria, CA, USA), respectively. We used a standardized immunohistochemistry kit (HercepTest for Immunoenzymatic Staining; DAKO).

Immunohistochemistry. A Histofine kit (Nichirei, Tokyo, Japan), which uses the streptavidin–biotin amplification method, was used in this study. Antigen retrieval was carried out by heating the slides in an autoclave at 120°C for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0) for Runx2, ER, PR, HER2 and Ki-67 immunostaining. The dilutions of the primary antibodies used in this study are as follows: Runx2, 1/1000; ER, 1/50; PR, 1/30; HER2, 1/200; and Ki-67, 1/50. 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 haematoxylin. As a negative control, normal mouse, rabbit or goat IgG was used instead of the primary antibodies, and no immunoreactivity was detected in these sections (data not shown).

Statistical analysis. Immunoreactivity of Runx2 was detected in the nuclei and the labeling index (LI) was subsequently obtained. Briefly, Runx2 immunoreactivity was evaluated in the nuclei of more than 1000 carcinoma cells for each case, and the percentage of immunoreactivity (i.e. the LI) was subsequently determined. In breast carcinoma cells, Runx2, ER, PR and Ki-67 immunoreactivity was detected in the nucleus, and the immunoreactivity was evaluated as a LI in the same was as described above. Cases with ER, PR or Ki-67 of more than 10% were considered positive in this study, according to a report on ER.[41] HER2 immunoreactivity was evaluated according to a grading system proposed in HercepTest (DAKO), and moderately or strongly circumscribed membrane staining of HER2 in more than 10% of carcinoma cells was considered positive.[42] An association between Runx2 immunoreactivity and clinicopathological factors of breast carcinoma patients was statistically evaluated using a correlation coefficient (r) and regression equation, Student's t-test, or a one-way ANOVA and Bonferroni test. Overall and disease-free survival curves were generated according to the Kaplan–Meier method, and the statistical significance was calculated using the log-rank test evaluating 64 cases with the Runx2 LI ≧37% (median value) as Runx2 positive and 56 cases with the Runx2 LI <37% as Runx2 negative in a group of non-distal metastatic breast cancer. Both univariate and multivariate analyses were evaluated by a proportional hazard model (COX) using PROC PHREG in SAS software (SAS Institute Inc., Cary, NC, USA).

Results

Immunohistochemistry of Runx2. Runx2 immunoreactivity was detected in the nuclei of breast carcinoma cells (Fig. 1a,b), and the mean value of the Runx2 LI in 137 breast carcinoma tissues examined was 43.1% (range, 0–99%) in total. Runx2 immunoreactivity was also detected in non-pathological myoepithelial and ductal cells (Fig. 1c). Ninety-five cases are ER positive (LI ≧ 10%) and 42 cases are ER negative (LI < 10%). Seventy-eight cases are PR positive (LI ≧ 10%) and 59 are PR negative (LI < 10%). The mean value ± SD of the Ki-67 LI in 137 breast carcinoma tissues examined was 21.3 ± 17.9% (range, 0–82%) in total.

Correlation of the Nucleus Runx2 LI with the clinicopathological factors of the cases examined. Table 1 summarizes the correlation of the Runx2 LI in breast carcinoma cells with the clinicopathological parameters in the breast carcinoma cases. Significant association between the Runx2 LI and stage ($P = 0.0004$), histological grade ($P = 0.046$) and HER2 status ($P = 0.002$) of the patients was demonstrated, but there were no significant correlation between the Runx2 LI and age ($P = 0.78$), menopausal status ($P = 0.69$) and lymph node status ($P = 0.66$) of the cases examined. The Runx2 LI tended to be correlated with ER ($P = 0.13$) and PR status ($P = 0.06$), but the correlation did not reach statistical significance. The Runx2 LI also correlated with both clinical stages and histological grades of the patients.

Correlation between the Runx2 LI and clinical outcome in 120 non-distal metastatic breast carcinoma patients (stage I–III). A significant association was detected between the Runx2 LI and recurrence ($P = 0.01$) or overall survival ($P = 0.003$) of the

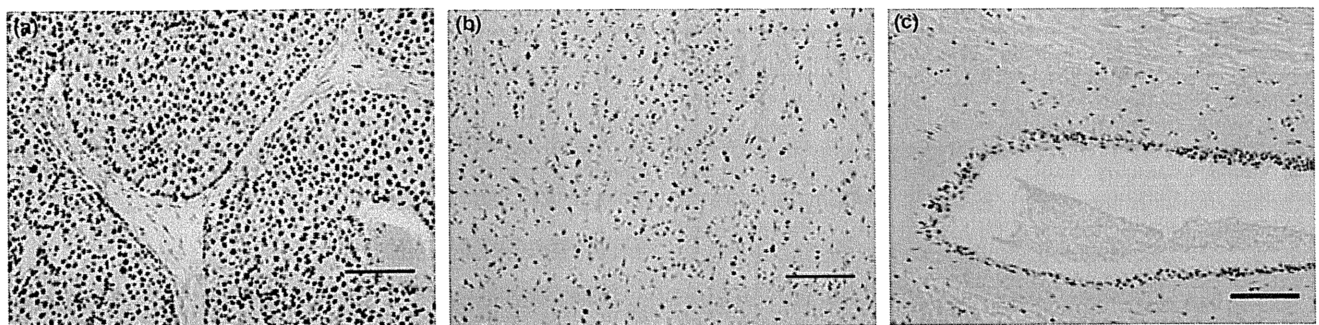

Fig. 1. Immunohistochemistry for Runx2. Runx2 immunoreactivity was detected in the nuclei of both carcinoma and stroma cells. (a) Case I with Stage III showed a labeling index (LI) = 97.0. (b) Case II with Stage II showed a LI = 37.0. (c) Normal mammary epithelial cells also include nucleus Runx2 positive. Bar, 100 μm.

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Table 1. Summary of an association between the nuclear Runx2 LI of carcinoma cells and the clinicopathological parameters in 137 breast cancers

	n	Runx2 LI	*P* value
Age (22–81 years)	137		0.78 ($r = -0.024$)
Menopausal status			
Premenopausal	50	43.1 ± 4.1	0.69
Postmenopausal	87	45.1 ± 2.9	
Stage			
I	34	35.3 ± 4.6	**0.0004**
II	67	42.0 ± 3.4	
III	19	47.2 ± 5.1	
IV	17	68.8 ± 5.7	
Tumor size			
<2.0 cm	18	49.8 ± 6.3	0.68
≥2.0 cm	94	46.5 ± 3.0	
Lymph node status			
Positive	17	45.5 ± 3.6	0.66
Negative	120	43.4 ± 3.2	
Distant metastasis			
Positive	60	68.8 ± 5.7	**<0.0001**
Negative	77	40.9 ± 2.5	
Histological grade			
1 (well)	27	34.7 ± 5.6	**0.046**
2 (moderate)	63	43.4 ± 3.6	
3 (poor)	47	51.2 ± 3.6	
ER status			
Positive	95	41.6 ± 2.9	0.13
Negative	42	49.3 ± 3.8	
PR status			
Positive	78	40.3 ± 3.2	0.06
Negative	59	49.2 ± 3.4	
HER2 status			
Positive	30	58.3 ± 5.4	**0.002**
Negative	107	40.5 ± 2.5	
Ki-67 LI (0–82%)			**0.004** ($r = 0.25$)

Data considered significant ($P < 0.05$) in the univariate analysis are shown in bold. Significant values were examined in the multivariate analysis in the present study. ER, estrogen receptor; LI, labeling index; PR, progesterone receptor.

patients in 120 breast carcinoma patients at stage I, II and III (Fig. 2a,b). In particular, among these 120 patients, ER negative cases (23 Runx2 positive and 20 Runx2 negative), the Runx2 LI was markedly associated with an increased risk of clinical recurrence ($P = 0.03$) (Fig. 2c) and overall survival of the patients (P not calculated because no patients died in the ER negative/Runx2 positive group) (Fig. 2d). However, among the ER-positive cases (41 Runx2 positive and 36 Runx2 negative), no significant association was detected between the Runx2 LI and an increased risk of recurrence ($P = 0.55$) (Fig. 2e) and overall survival ($P = 0.39$) (Fig. 2f).

In a Univariate analysis, the Runx2 LI evaluated as a continuous variable also turned out to be a significant prognostic factor ($P = 0.049$ in disease-free survival and $P = 0.004$ in overall survival), and an independent prognostic factor when it was included in a multivariate analysis instead of the dichotomized variable ($P = 0.01$ and $P = 0.04$, respectively) (Table 2). Because no cases had received administration of trastuzumab agent in this study, HER2 positive was a remarkably poor prognostic factor.[43]

Discussion

In the present study, the Runx2 LI in breast carcinoma cells was significantly associated with stage ($P = 0.0004$) and histological grade ($P = 0.046$) of the patients examined. These findings indicate the possible roles of Runx2 in the biological behavior of breast carcinoma patients, including those without metastasis. We also demonstrated that the prognosis or clinical outcome of cases associated with a high Runx2 LI is generally poor. In particular, in 120 cases not associated with distant metastasis, a significant positive association was detected between the Runx2 LI and both the risk of recurrence and overall survival of patients. Furthermore, this association was more pronounced in the group of 43 ER-negative cases (36%). This group of ER-negative carcinoma included HER2 positive and basal-like subgroups of breast carcinoma. These findings also suggested that Runx2 could serve as a marker of aggressive biological behavior and its inhibition might open a new strategy of therapy for these cases.

Breast cancer development consists of many sequential steps, including primary tumor growth, neovascularization around the tumor, invasion, extravasation and subsequently formation of bone metastasis.[24] Many *in vitro* studies demonstrated that Runx2 might participate in these steps in multiple fashions. Regulation or modification of VEGF secretion by Runx2 was reported in neovascularization.[44] Regulation of several MMP secretion by Runx2 was also postulated to be linked with subsequent invasion of carcinoma cells.[45,46] Runx2 was proposed to subsequently mediate PTHrP expression of metastatic breast carcinoma cells in the microenvironment of bone and might be involved in the formation of a vicious cycle.[2] All of the above might be related to an adverse clinical outcome for patients but little has actually been demonstrated in clinical cases of human breast carcinoma. In the present study, we demonstrated a significant correlation of the status of Runx2 expression in carcinoma cells with the histological grade and stage of patients. In addition, the possibility of potential involvement of Runx2 in earlier phases of breast cancer development was also raised in the present study.

In the present study, the groups of patients with elevated Runx2 expression were significantly associated with a poor prognosis in the ER-negative group of patients, while this association was not detected in the ER-positive carcinoma patients. In our cohort of ER-positive breast cancer patients, 34 of 95 cases (35.8%) received administration of tamoxifen, while in that of the ER-negative breast cancer patients, six of 42 cases (14.3%) did. Estrogenic depletion affects not only breast carcinoma cells but also the entire body of patients. It is true that SERM can prevent the systemic effects of estrogen depletion to some extent, but ovarian suppression and administration of AI result in marked side-effects, especially in the skeletal system causing development of clinically significant osteoporosis in some patients.[36–39]

Results of various *in vitro* studies have shown that suppression of estrogenic activation caused maturation of osteoclasts in a direct manner.[47] Estrogen, acting via ER, causes upregulation of Fas ligand (FasL) in osteoclast progenitors (pOC) and/or OC themselves.[47] The increased FasL levels cause apoptosis because these OC lineage cells also express Fas. Fas ligand expression is also reported to be diminished or even suppressed without estrogens in these systems, and the life span of OC was actually reported to be elongated in the absence of estrogen in a culture medium. These OC might stimulate osteoblasts to form bone via poorly defined factors but the resorptive effects of these OC are usually considered dominant.[47]

Results of several reported studies suggest that anti-estrogen therapy did amplify the maturation of OC, resulting in the development of osteoporosis. Osteoporosis is a disease of increased bone turnover, in which the bone-resorbing activity of OC outpaces the bone-forming activity of osteoblasts, resulting in the loss of predominantly trabecular bone.[47] Both of these cell types are reported to respond to estrogen, but results of previous studies suggest that the response of human bone to estrogen

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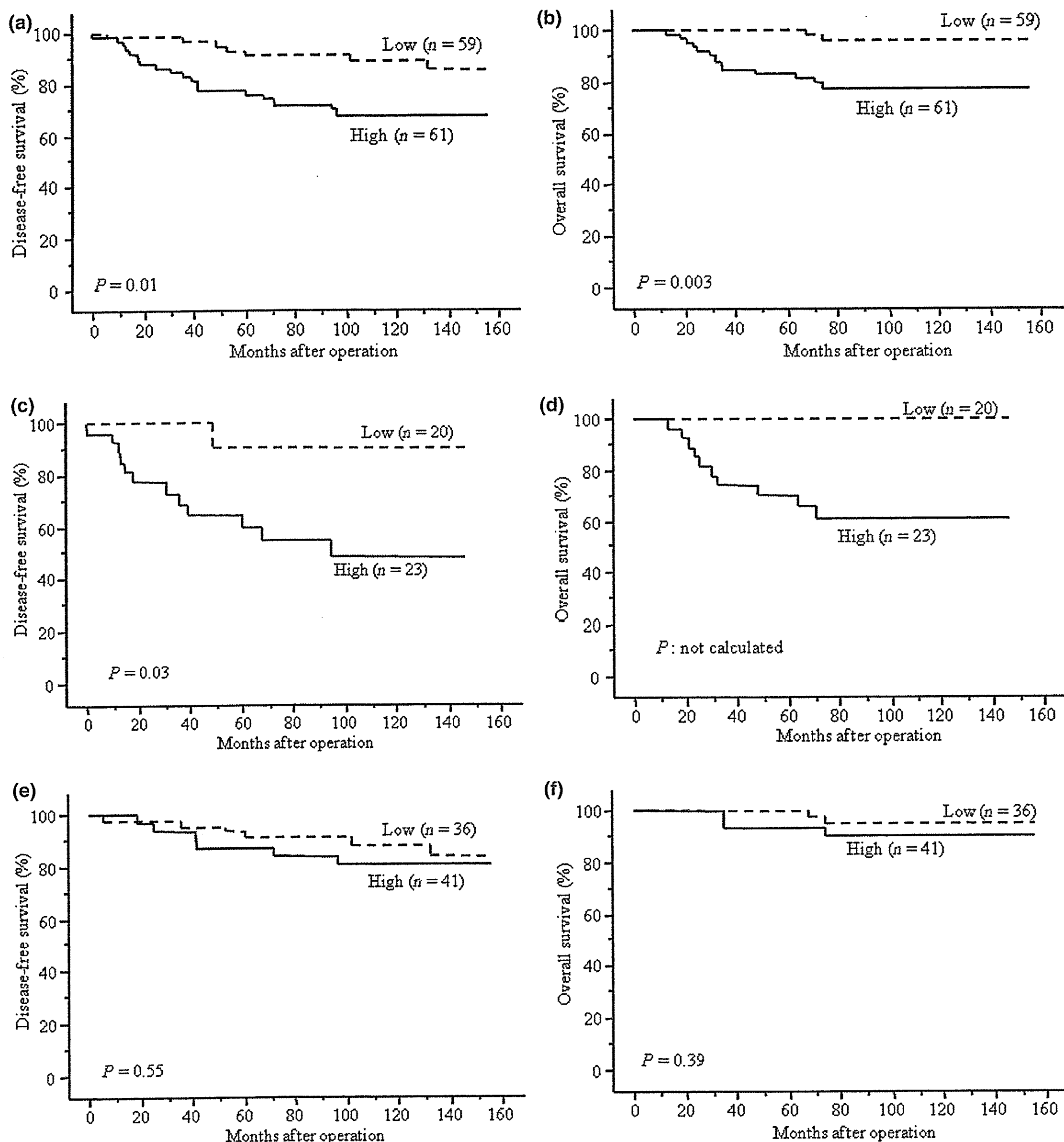

Fig. 2. Disease-free (a) and overall (b) survival of 120 cases associated with non-distal metastatic breast carcinoma according to nucleus Runx2 immunoreactivity (Kaplan–Meier method), respectively. Disease-free (c) and overall survival (d) of 77 estrogen receptor (ER)-positive breast carcinoma cases associated with non-distal metastatic breast carcinoma according to nucleus Runx2 immunoreactivity, respectively. Disease-free (e) and overall survival (f) of 43 ER negative breast carcinoma cases associated with non-distal metastatic breast carcinoma according to nucleus Runx2 immunoreactivity, respectively.

withdrawal is at least in part mediated by a network of inflammatory and osteoclastogenic cytokines, including tumor necrosis factor α (TNFα) and interleukin-1 (IL-1), released by stromal/osteoblast lineage cells and T cells.[48,49]

Decreased levels of estrogens usually result in increased production of the cytokine IL-7 by osteoblasts, which stimulates proliferation of T cells and their secretion of both TNFα and receptor activator of NF-κB Ligand (RANKL).[49] Tumor necrosis factor α stimulates osteoblasts to increase their synthesis of RANKL, which results in the differentiation and activation of OC. Tumor necrosis factor α also acts directly on pOC, synergizing with RANKL for OC differentiation.[49] Additional pro-osteoclastogenic cytokines and growth factors are also expressed in T cells and other peripheral blood mononuclear cells.[49,50]

It is practically very difficult to confirm that bone-metastatic breast cancer cells are also associated with elevated Runx2 expression in the cases with high Runx2 expression in primary breast carcinoma cells because clinically the availability of specimens for both primary and bone metastasis are in general rare. However, metastatic breast carcinoma cells associated with high Runx 2 expression may facilitate the process of osteoporosis in the bone microenvironment by RANKL secretion of osteoblasts via PTHrP secretion,[30] but further investigations are required for clarification.

HER2 status of patients turned out to be a strong independent factor because administration of Herceptin had not been used in any of the patients examined in this retrospective study. Runx2 LI in carcinoma cells was also markedly correlated with the

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Table 2. Summary of univariate and multivariate analyses of overall survival in 137 breast carcinoma patients

Variable	Univariate	Multivariate	
	P-value	*P*-value	Relative risk (95% CI)
Disease-free survival			
Lymph node status (positive/negative)	**0.001**	0.001	7.1 (2.4–21.5)
HER2 status (positive/negative)	**0.002**	0.002	4.0 (1.6–10.1)
Runx2 LI (99–0%)	**0.049**	0.01	1.02 (1.01–1.1)
Ki-67 LI (82–0%)	0.49		
Histological grade (3/1, 2)	0.56		
Tumor size (≥2.0 cm/< 2.0 cm)	0.96		
Overall survival			
HER2 status (positive/negative)	**0.001**	0.02	4.0 (1.2–13.2)
Runx2 LI (99–0%)	**0.004**	0.04	1.03 (1.001–1.1)
Histological grade (3/1, 2)	**0.01**	0.5	
Lymph node status (positive/negative)	**0.03**	0.02	4.2 (1.1–1.68)
Ki-67 LI (82–0%)	**0.048**	0.29	
Tumor size (≥2.0 cm/<2.0 cm)	0.51		

Data considered significant ($P < 0.05$) in the univariate analysis are shown in bold. CI, confidence interval; LI, labeling index.

HER2 status of patients ($P = 0.002$). The correlation of HER2 over-expression with Runx2 expression has not been demonstrated in any human carcinoma and it awaits further investigation for clarification.

In the present study, we showed that the Runx2 LI of breast carcinoma cells associated with clinical stage, histological grade and HER2 status and High Runx2 LI is a poor prognostic factor. Runx2 is currently considered one of the essential regulators of both skeletal development and progression of several tumors including breast and prostate tumors. These tumor cells have been known to frequently metastasize to the bone.[51] However, the potential roles on primary carcinoma and bone metastasis still remain relatively unknown. Further investigation into the potential roles of Runx2 in these interactions should provide paths toward the establishment of effective management or control of bone metastasis with breast carcinoma patients.

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Disclosure Statement

The authors have no conflict of interest.

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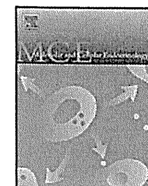

Review

Steroid sulfatase and estrogen sulfotransferase in human carcinomas

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ABSTRACT

Estrogens are closely involved in the development of hormone-dependent carcinomas. Estrone is locally produced from circulating inactive estrone sulfate by steroid sulfatase (STS), while estrone is inversely inactivated into estrone sulfate by estrogen sulfotransferase (EST). Recent studies suggested importance of this STS pathway in various human carcinomas. Therefore, in this review, we summarized recent results of STS and EST in several estrogen-dependent carcinomas. STS and EST expressions were detected in the breast and endometrial carcinomas, and activation of STS pathway due to increment in STS and/or decrement in EST expressions plays important role in their estrogen-dependent growth. STS expression was also reported in the ovarian and prostate carcinomas. STS/EST status was associated with intratumoral estrogen level in the colon carcinoma, and STS-negative/EST-positive colon carcinoma patients had longer survival. Therefore, STS pathway and estrogen actions may play an important role in the development of these carcinomas, and further investigations are required.

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1. Introduction

It is well known that estrogens are closely involved in the development of hormone-dependent carcinomas. Estrogens actions are mediated through an interaction with the estrogen receptors (ERs). ER is expressed in a great majority of the breast carcinoma, the most representative estrogen-dependent malignancy. However, a great majority of breast carcinomas arise after menopause when ovaries ceased to be functional, and ER expression is not necessarily associated with plasma concentrations of estrogens in these patients.

Recent studies have demonstrated that estrogens are locally produced from circulating inactive steroids in the breast carcinomas by steroid sulfatase (STS) and aromatase (Suzuki et al., 2005) (Fig. 1). The former pathway converts to estrone from circulating estrone sulfate by STS, while estrone is inversely inactivated into estrone sulfate by estrogen sulfotransferase (EST). In the latter, aromatase produces estrone from circulating androstendione. Estrone is subsequently converted to a potent estrogen, estradiol, by reductive 17β-hydroxysteroid dehydrogenases (17βHSDs). 14 isozymes of 17βHSD have been currently identified, and 17β-reduction (17βHSD1, 7, 12 etc) or oxidation (17βHSD2, 4, 14 etc)

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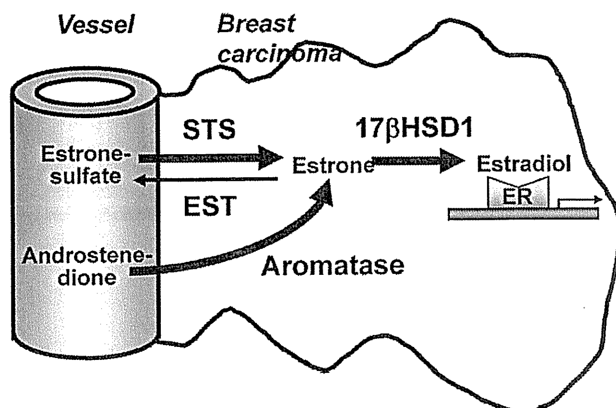

Fig. 1. Scheme representing *in situ* production of estrogens in the breast carcinoma tissue which is currently postulated. STS; steroid sulfatase, EST; estrogen sulfotransferase, 17βHSD1; 17β-hydroxysteroid dehydrogenase type 1, and ER; estrogen receptor.

of estrogens is catalyzed by different 17βHSD isozymes. The reductive 17βHSD pathway is dominant in the breast carcinoma, and 17βHSD1 enzyme is considered to play the most important role in the enhanced conversion of estrone into estradiol in the breast carcinoma (Nagasaki et al., 2009).

Estradiol concentration in the breast carcinoma tissue was not significantly different regardless of the menopausal status of patients (Pasqualini, 2004), and a large proportion, *i.e.*, approximately 75% before menopause and close to 100% after menopause, of biologically active estrogens is considered to be locally produced in the breast carcinoma (Labrie et al., 2003). Comparison of STS and aromatase activities has been previously examined in the breast carcinoma by several groups. For instances, Santner et al. (1984) found that as much as 10-fold more estrone could originate from estrone sulfate *via* the STS pathway than from androstenedione by the aromatase route, and Pasqualini et al. (1996) showed that STS activity measured by estrone production from estrone sulfate was 50–200 times greater than aromatase activity evaluated by testosterone as a substrate. These data are suggestive of relative importance of STS pathway in the pathogenesis of the breast carcinoma (Reed et al., 2005).

ER is expressed in various human carcinoma tissues, including endometrial, ovarian, prostate and colon carcinomas, in addition to breast carcinoma. Its significance, however, has not been fully elucidated, because some carcinomas are not necessarily considered targets for estrogens. However, an increasing body of evidence suggests that *in situ* production of estrogen through the STS pathway may play an important role in the development of these carcinomas, and the presence of STS may lead to therapeutic potential in a selective group of the carcinomas. In order to obtain a better understanding of local actions of estrogens, it becomes very important to examine the status of STS and EST in these carcinomas. Therefore, in this review, we summarized results of recent studies on STS and EST in several estrogen-dependent carcinomas, and discuss the significance of STS pathway in these carcinomas.

2. STS and EST

2.1. STS

Sulfatase family catalyzes hydrolysis of sulfate ester bonds of a wide variety of substrates, and 17 human sulfatase proteins and their corresponding genes have been identified (Ghosh, 2007). STS belongs to the sulfatase family, and also named as arylsulfatase C (ARSC). STS hydrolyzes several sulfated steroids, such as estrone sulfate and dehydroepiandrosterone (DHEA) sulfate (Reed et al., 2005). STS is a microsomal enzyme and is an integral membrane protein of endoplasmic reticulum. Human *STS* gene is localized on the X-chromosome and mapped in Xp22.3-Xpter, and consists of 10 exons (Reed et al., 2005). STS is expressed in several tissues including the placenta and skin (Ghosh, 2007; Selcer et al., 2007), although it is also true that STS immunoreactivity was negligible in many human tissues (Miki et al., 2002). Mutations in the *STS* gene and subsequent inactive enzyme have been associated with X-linked ichthyosis (Ghosh, 2004; Reed et al., 2005). Several single nucleotide polymorphisms (SNPs) have been reported in the promoter region and exons of *STS* gene (Goodarzi et al., 2007; Udler et al., 2009; Matsumoto et al., 2010). Some of these are suggested to possibly regulate the transcriptional and/or enzymatic activities of STS (Matsumoto et al., 2010), but their significance remains unclear.

Six different promoters were detected to drive STS expression, giving rise to transcripts with unique first exons (Valle et al., 2006, 2007), and exon 1a was associated with the promoter that drives expression in the placenta. In addition, Nardi et al. (2009) reported two additional alternatively spliced transcripts in human tissues and cell lines. On the other hand, Zaichuk et al. (2007) demonstrated that STS transcription was up-regulated by estradiol through the binding of ER to estrogen responsive elements (EREs) located in the promoter regions, which resulted in driving 1a and 1b transcripts in the breast carcinoma. Therefore, STS expression may be regulated by different promoters according to the cellular functions and/or tissues. Interleukin (IL) 6 and tumor necrosis factor (TNF) α were reported to stimulate STS activity in breast carcinoma cells, possibly *via* a post-transcriptional modification of the enzyme (Newman et al., 2000).

Tsunoda et al. (2006) found that serum levels of estrogens, such as estrone, estrone sulfate, estradiol, and estradiol sulfate, were decreased after operation in postmenopausal patients with markedly STS positive breast carcinoma. In addition, Kirilovas et al. (2007) reported that STS activity in ovarian tumors was correlated with the serum estradiol level. These findings may suggest that intratumoral STS activity partly affects plasma estrogen concentration. However, human liver represents a major site for metabolism of circulating estrogens, and further examinations are required.

2.2. EST

The cytosolic sulfotransferases (SULTs) catalyze the transfer of the sulfuryl group to an acceptor substrate including phenols, estrogens, hydroxysteroids, arylamines, primary alcohols and iodothyronines. In human, three families of SULTs have been characterized: SULT1 family included 8 subfamilies (A1, A2, A3, A4, B1, C2, C4, and E1), SULT2 family included 2 subfamilies (A1 and B1), and SULT4 family (A1) (Pasqualini, 2009). Among these SULTs, SULT1E1 has the most marked sulfating activity for estrogens (Adjei et al., 2003; Hui et al., 2008). Therefore, SULT1E1 regulates the STS pathway and subsequently local estrogen levels in human tissues, and termed EST. In addition, EST is the only sulfotransferase that displays affinity for estradiol in a physiological concentration range (Zhang et al., 1998; Pasqualini, 2009).

EST gene is localized on chromosome 4q13.2, and EST protein consists of 294 amino acids. Miki et al. (2002) demonstrated that EST mRNA expression was detected in various human tissues, and suggested that EST was involved in protecting peripheral tissues from possible excessive estrogenic effects. To date, more than 20 SNPs have been found in *EST* gene (Iida et al., 2001; Adjei et al., 2003), and SNP IVS4-1653 (rs3775775) was significantly associated with increased recurrence of breast carcinoma (Choi et al., 2005). A positive association between EST SNP and risk of endometrial cancer has been also reported (Rebbeck et al., 2006; Hirata et al., 2008). EST expression is controlled by progesterone in nor-

mal endometrium (Dassen et al., 2007), but little is currently known on the regulatory mechanism of EST.

3. STS and EST in the breast carcinoma

Estrone sulfate has a relatively long half-life in the peripheral blood, and the level of estrone sulfate is 5–10 times higher than that of unconjugated estrogens such as estrone, estradiol and estriol, during the menstrual cycle and in postmenopausal women (Pasqualini, 2004). Tissue concentration of estradiol in the breast carcinoma was 5-fold higher than in plasma in premenopausal women, and 23-fold higher in postmenopausal women (Pasqualini, 2004). Estradiol concentration was also 2-fold higher in breast carcinoma tissues than in the areas considered as morphologically normal (Chetrite et al., 2000). STS mainly catalyzes estrone sulfate to estrone in breast carcinoma (Fig. 1), which immensely contributes to the intratumoral estrogen production.

STS enzyme activity was detected in the great majority of breast carcinomas (Evans et al., 1994). STS mRNA expression was higher in breast carcinoma tissues than that in normal breast tissues, and it was significantly associated with the breast carcinoma progression and poor clinical outcome of the patients (Utsumi et al., 1999; Miyoshi et al., 2003; Suzuki et al., 2009). Irahara et al. (2006) reported that both STS and 17βHSD1 mRNAs were up-regulated in soft tissue metastases of breast carcinoma compared to those in primary tumors, suggesting importance of STS associated with local estrogen production in metastatic sites of the breast carcinoma. STS immunoreactivity was detected in carcinoma cells in 60–90% of breast carcinoma cases (Saeki et al., 1999; Suzuki et al., 2003; Yamamoto et al., 2003; Tsunoda et al., 2006) (Fig. 2(A)). STS immunoreactivity was frequently detected in postmenopausal patients (Tsunoda et al., 2006), and was significantly associated with tumor size and increased risk of recurrence (Suzuki et al., 2003). Several groups are currently developing STS inhibitors, and results of a phase I study suggested that STS inhibitor may be effective in estrogen-dependent breast carcinomas including those progressed on aromatase inhibitors (Stanway et al., 2006).

Expression of STS mRNA was significantly higher in ductal carcinoma *in situ* (DCIS) than the non-neoplastic breast tissue, and STS immunoreactivity was detected in 54% of DCIS tissues (Shibuya et al., 2008). Therefore, *in situ* production of estrogens by STS may also play important roles in DCIS as a precursor lesion of invasive ductal carcinoma.

The concentration of estrone sulfate was significantly (approximately 10 times) higher in breast carcinoma tissues than that in plasma levels (Pasqualini, 2004), and EST enzyme activity was detected in breast carcinoma tissues and normal breast tissues (Tseng et al., 1983). MCF-7 breast carcinoma cells transfected with EST demonstrated much lower estrogen-stimulated DNA synthesis or cell proliferation than control MCF-7 cells that did not possess EST (Qian et al., 1998; Falany et al., 2002). In addition, Fu et al. (2009) examined EST mRNA expression in MCF-10A-derived lineage cell culture model, and reported that EST was abundantly expressed in FCF-10A and preneoplastic MCF-10AT1 cell lines, but was markedly repressed in neoplastic MCF-10A-derived cell lines as well as in MCF-7 cells. Therefore, the loss of EST expression during the process of carcinogenesis may represent a possible important factor in increasing local estrogen production in the breast carcinoma as well as the induction of STS expression.

EST enzyme activity and its mRNA expression were detected in breast carcinoma tissues (Tseng et al., 1983; Suzuki et al., 2003; Yoshimura et al., 2004; Suzuki et al., 2009). EST mRNA expression was detected in intratumoral stromal cells and adipocytes adjacent to the carcinoma in addition to the breast carcinoma cells. Suzuki et al. (2003, 2009) reported that EST mRNA level was high in non-malignant breast tissues. EST immunoreactivity was detected in the carcinoma cells in 40% of invasive breast carcinomas by Suzuki et al. (2003) or 80% of those by Hudelist et al. (2008), and it was also present in the epithelial cells of normal mammary glands (Suzuki et al., 2003) and DCIS (Hudelist et al., 2008) (Fig. 2(B)). EST immunoreactivity was inversely correlated with tumor size or lymph node status in the invasive breast carcinoma, and was also significantly associated with a decreased risk of recurrence or improved prognosis of the patients (Suzuki et al., 2003). These findings may be due to the fact that EST-negative breast carcinomas result in an increment of intratumoral estrogen concentrations, which subsequently results in an increased recurrence and/or poor prognosis in these patients.

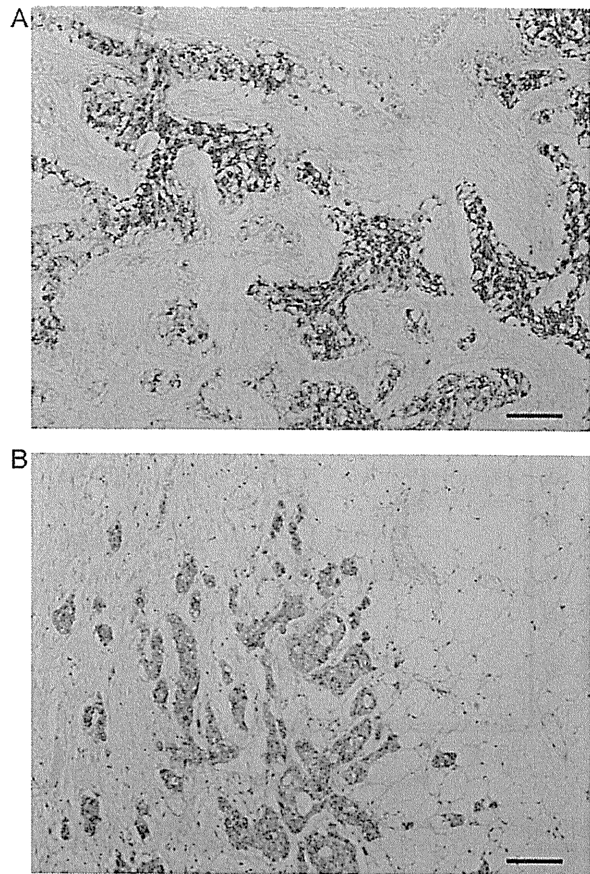

Fig. 2. STS (A) and EST (B) immunoreactivities in the breast carcinoma. Both immunoreactivities were detected in the cytoplasm of carcinoma cells. Bar = 100 μm, respectively.

Because sulfated steroid conjugates, such as estrone sulfate, carry a net negative charge at physiological pH, their transfer across cell membranes is considered to be carrier mediated. Sulfated steroids have been identified as substrates for a superfamily of organic anion transporting polypeptides (OATPs) (Hagenbuch and Meier, 2003). Miki et al. (2006) demonstrated that OATP-A was a target gene of steroid and xenobiotic receptor (SXR) and SXR immunoreactivity was positively correlated with STS immunoreactivity in the breast carcinoma, suggesting that estrone sulfate may be transported into breast carcinoma cells by OATP-A and subsequently transformed into estrone by STS. In addition, Muto et al. (2007) reported that other member of OATPs, liver-specific organic anion transporter-2 (LST-2), expressed in the breast carcinoma, and its immunoreactivity was significantly associated with a decreased risk of recurrence and improved prognosis in 102 breast carcinoma cases. When we further analyzed an association between LST-

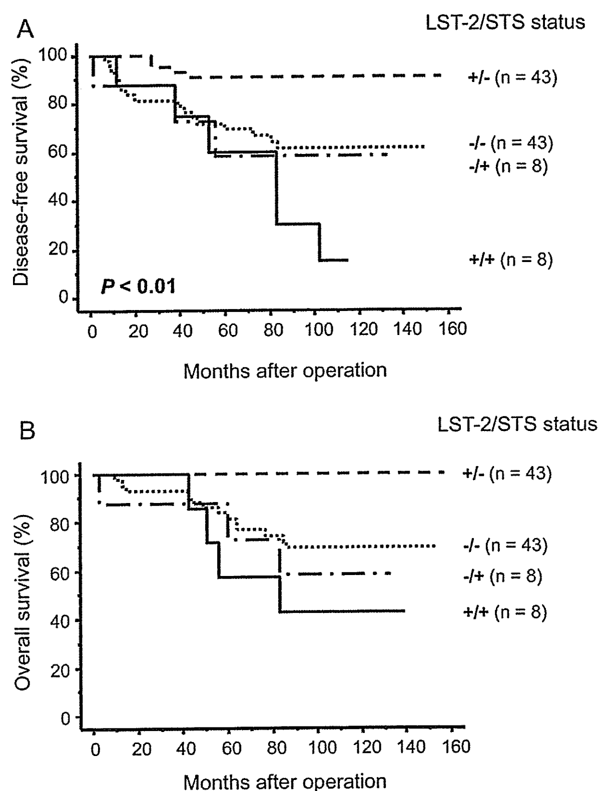

Fig. 3. Disease-free (A) and overall (B) survival of 102 patients with breast carcinoma according to LST-2/STS status (Kaplan–Meier method). LST-2/STS status was evaluated by immunohistochemistry. A+/+ group (*i.e.*, breast carcinoma positive for both LST-2 and STS) was significantly associated with an increased risk of recurrence ($P < 0.01$ by a log-rank test). Similar tendency was detected in the overall survival (B), although *P* value was not calculated because no patient had died in the +/− group in this study. Original data were taken from Muto et al. (2007).

2/STS immunohistochemical status and clinical outcomes of these patients, a breast carcinoma group positive for both LST-2 and STS did demonstrate the worst prognosis (Fig. 3). Therefore, LST-2 may be an important regulator for STS pathway in the breast carcinoma by possibly increasing intracellular concentration or availability of the precursor estrone sulfate.

Intratumoral estrogen levels are regulated by complex enzyme systems in the breast carcinoma (Fig. 1). Recently, Lønning et al. (2009) demonstrated a significantly increased estradiol concentration in ER-positive breast carcinoma compared to normal tissue, but not in ER-negative cases, while the estrone level was significantly reduced in the carcinoma tissue regardless of ER status. On the other hand, intratumoral concentration of estrone sulfate was significantly higher in ER-positive postmenopausal breast carcinoma and ER-negative breast carcinoma than the normal tissue. Therefore, high concentration of estradiol in the breast carcinoma may be caused by not only enhanced local estrogen production but also alternative mechanisms as receptor ligand binding or alternation of intratumoral estrogen dynamics including the enhanced conversion of estrone to estradiol by 17βHSD system (Lønning et al., 2009).

4. STS and EST in other carcinomas

4.1. Endometrial carcinoma

Endometrium is one of the target tissues of estrogen, and endometrial carcinoma is known as an estrogen-dependent malignancy as well as the breast carcinoma. Endometrial carcinoma frequently expresses ER, and most cases occur in postmenopausal women. Estradiol level in the endometrial carcinoma tissue was significantly higher than normal endometrium (1.9-fold in premenopausal patients, and 1.5-fold in postmenopausal patients (Berstein et al., 2003)), and high concentration of estrogens is maintained in endometrial carcinoma tissues regardless of menopausal status of the patients.

STS immunoreactivity was negative in the normal endometrium, but that of EST was detected in glandular epithelial cells in the secretory phase (Utsunomiya et al., 2004). STS activity was significantly higher in the endometrial carcinoma than in normal endometrial tissues (Abulafia et al., 2009). Smuc and Rizner (2009) however, recently reported that EST mRNA expression in endometrial carcinoma was significantly decreased compared to the paired normal endometrium, whereas STS mRNA level was not significantly different between these groups. Both STS and EST immunoreactivities were detected in 86% and 29% of endometrial carcinomas, respectively, and STS/EST ratio was significantly associated with poor prognosis of the endometrial carcinoma patients (Utsunomiya et al., 2004). Therefore, a possible activation of STS pathway due to increment in STS and/or decrement in EST expressions may also play important roles in the estrogen-dependent growth of endometrial carcinoma, as in breast carcinomas. Recently, Foster et al. (2008) demonstrated an inhibition of endometrial carcinoma growth by STS inhibitor using a hormone-dependent endometrial xenograft model in ovariectomized mice.

4.2. Ovarian carcinoma

Ovarian carcinoma is mainly originated from the surface epithelium, and has poor prognosis because of frequent occult metastasis in the peritoneal cavity and detection at the advanced clinical stage. Previous studies have suggested an association between estrogens and initiation and/or progression of ovarian carcinoma, although some inconsistent findings have been reported. This hypothesis is partially supported by recent large population-based case–control study and meta-analysis of estrogen-based hormone replacement therapy (HRT), in which incidence of ovarian carcinoma was statistically increased in both current and ever users (O'Connor, 2006; Ragonese et al., 2006).

STS enzymatic activity was also detected in the ovarian carcinoma (Milewich and Porter, 1987). Chura et al. (2009) demonstrated that ovarian cancer patients with higher STS activity were significantly associated with shorter progression-free survival than those with STS lower activity. STS immunoreactivity was detected in 70% of ovarian clear cell adenocarcinoma (Okuda et al., 2001). Very recently, Day et al. (2009) demonstrated that STS enzymatic activity in ER-positive ovarian carcinoma cell line OVCAR-3 was inhibited by STS inhibitor STX64, suggesting its therapeutic potential also for ovarian carcinoma. It awaits further investigation to clarify the importance of STS pathway in the ovarian carcinoma.

4.3. Prostate carcinoma

It is well known that androgens play a pivotal role in the pathogenesis of prostate carcinoma through androgen receptor. STS catalyzes DHEA from DHEA sulfate, and it possibly play important role in the intratumoral androgen production (Day et al., 2009). Estrogens are, however, generally considered an important factor associated with cell proliferation of prostate carcinoma cells (Härkönen and Mäkelä, 2004).

STS activity in prostate carcinoma cells produced significant levels of estrone and estradiol when these cells were treated with estrone sulfate (Nakamura et al., 2006). Giton et al. (2008) demon-

strated that serum estrone sulfate concentration was significantly higher in prostate cancer patients than that from age-matched control, and was significantly higher in prostate carcinoma patients associated with a poor prognosis. They suggested that higher serum estrone sulfate would lead to an increment of local estrogen production in the prostate carcinoma by STS pathway. Kapoor and Sheng (2008) examined biological functions of EST in the prostate carcinoma cells, and demonstrated that EST significantly repressed ERE activation by estradiol through ER.

STS immunoreactivity was not detected in non-neoplastic prostate tissues (Nakamura et al., 2006), while EST mRNA and protein were detected in the benign prostatic hyperplasia (Takase et al., 2007). Both STS and EST mRNA was detected in prostate carcinoma tissues, and STS and EST immunoreactivities were positive in 85% and 75% of prostate carcinomas, respectively (Nakamura et al., 2006). Therefore, STS and EST may regulate *in situ* production and action of estrogens in the prostate carcinoma tissues.

4.4. Colon carcinoma

A great majority of colon carcinomas expresses ERβ (Wong et al., 2005), and a number of observational studies (Grodstein et al., 1999; Newcomb et al., 2007) and randomized trials (Anderson et al., 2004) have demonstrated that HRT affects the incidence of colon cancer in postmenopausal women. Therefore, estrogens are considered to be involved in the pathogenesis of colon carcinoma, but the biological and/or clinical significance of estrogens has remained largely unclear in human colon carcinoma.

Recently, Sato et al. (2009) measured intratumoral concentrations of estrogens in colon carcinomas, and tissue concentrations of total estrogens (*i.e.*, estrone and estradiol) and estrone were significantly higher in colon carcinoma than in non-neoplastic colonic mucosa, and higher intratumoral concentrations of total estrogens and estrone were significantly correlated with poorer prognosis of the patients. In addition, intratumoral concentration of total estrogens in the colon carcinoma was significantly associated with STS/EST immunohistochemical status, but not with aromatase immunoreactivity (Sato et al., 2009). Immunoreactivities for STS and EST were detected in 61% and 44% of the colon carcinomas, and STS-negative and EST-positive colon carcinoma patients had significantly longer survival (Sato et al., 2009). Therefore, estrogens are postulated to be locally produced by STS pathway and may contribute to the progression of colon carcinoma, and STS inhibitors may be clinically effective in a selective groups of colon carcinoma patients. However, these findings are not necessarily consistent with previous reports that HRT reduced the risk of colon cancer (Grodstein et al., 1999) and improved survival from colon cancer (Mandelson et al., 2003). Therefore, further examinations are required to clarify the significance of estrogen and STS pathway in the colon carcinoma.

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ORIGINAL ARTICLE

Histopathological subclassification of triple negative breast cancer using prognostic scoring system: five variables as candidates

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Abstract We attempted to subclassify triple negative breast cancer (TNBC) cases into subgroups according to clinical outcome or prognosis of TNBC patients using archival specimens. We analyzed 102 Japanese cases of invasive TNBC who underwent surgery between January 1998 and December 2007. The clinicopathological factors and clinical information were retrospectively retrieved from reviewing the charts of the patients. Immunohistochemical staining was performed for estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor 1 (EGFR1), CK5/6, CK14, Ki-67, and CD31 for microvessel density (MVD). Median follow-up time of the patients was 68.5 months. Multivariable analysis demonstrated that pathologic node status was the most significantly associated with relapse-free survival (RFS) and breast cancer-specific survival (BCSS) of these patients. Pathological tumor size, basal-like type, Ki-67 labeling index (LI) and MVD were also independently associated with RFS and BCSS. Based on these results, we devised the risk score system reflecting hazard ratios of these prognostic factors above. With this system, TNBC patients in this study were classified into three subgroups (low-risk group: score 0–3, intermediate-risk group: score 4–7 and high-risk group: score 8–10). The significant difference of RFS and BCSS was detected among these three different subgroups of the patients ($p < 0.05$). We propose the risk score system, which incorporated pathologic nodal status, size of the primary tumor, the presence or absence of basal-like features, Ki-67 LI, and MVD in order to predict postoperative clinical course of the Japanese TNBC patients.

Keywords Triple negative breast cancer · Histopathology · Prognosis · MVD · Ki-67 · Basal-like

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Introduction

Breast cancer is a heterogeneous disease and has been recently proposed to be further classified into five different subtypes according to the results of gene expression profiling using DNA microarrays (luminal A, luminal B, human epidermal growth factor receptor 2 (HER2) over-expressing, basal-like and normal-like) [1, 2]. This analysis has been generally considered to be of clinical value because individual subtypes were associated with different clinical course or prognosis. Basal-like type has been reported to be associated with more unfavorable clinical course or outcome compared to luminal types and to be characterized by expression of myoepitherial/basal cytokeratins (CK5/6, CK14, and CK17) and epidermal growth factor receptor 1 (EGFR1) [3]. In addition, this type principally lacks the expression of estrogen receptor (ER), progesterone receptor (PR), and HER2 and has been also frequently termed triple negative breast cancer (TNBC) in routine clinical practice [3–5]. These two basal-like type and TNBC have a similar feature but the overlap is not