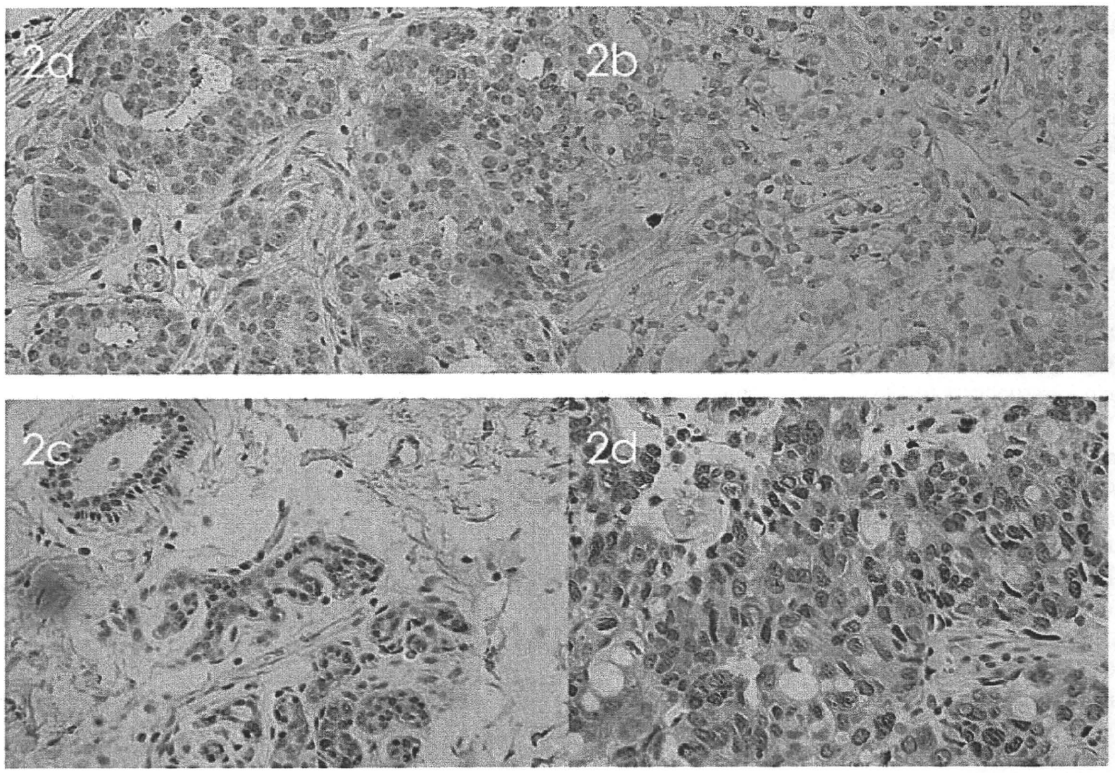


Figure 2. *Representative illustrations of HSP-70 IHC staining (400× magnification). Figure 2a (pre-treatment) and 2b (post-treatment) showed down-regulation of HSP-70 in patient #71 (responder), figure 2c (pre-treatment) and 2d (post-treatment) showed up-regulation of HSP-70 in patient #100 (non-responder).*

degree of reduction) than at the 12th week. The suppression of Ki-67 LI with the use of AI (anatrozole) was also reported to be correlated with a better recurrence-free survival (9). Results of our present study also demonstrated that the reduction of Ki-67 predicted the observed response to treatment with neoadjuvant AI, which is consistent with those in the previously reported studies. The pretreatment cut-off values of Ki-67 level have been in dispute but those of 10% appear to be widely accepted. The pretreatment high Ki-67 level of carcinoma, defined as >10%, was associated with decrement of Ki-67 and HSP-70 after completion of treatment, which was also found to be a significant predictor for treatment response of the patients as well. Results of these findings also suggest that endocrine therapy may still be effectively used in ER-positive cases associated with high cell proliferation but this awaits further investigations for clarification.

It is also important to evaluate wide-scale alterations of proteins or genes before and after neoadjuvant AI therapy. The expression patterns of various proteins underwent various changes following AI treatment: Ki-67, aromatase, ER-alpha, ER-beta, PgR, cyclin D1, p53, phosphorylated form of ER-alpha Ser118, ER-alpha Ser167, and p44/42 MAPK

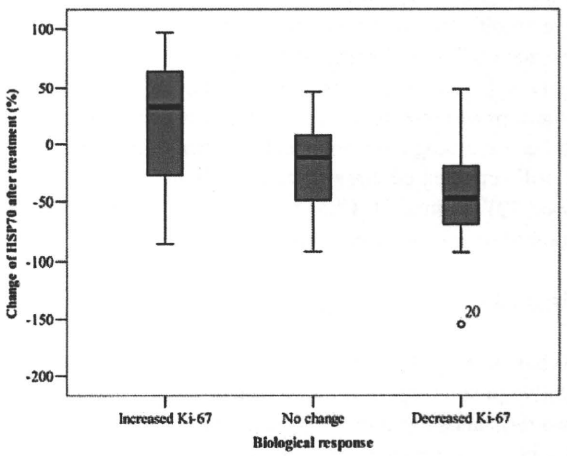


Figure 3. *Mean differences of HSP-70 among different categories of biological responders (p=0.22 between non-responders and responders).*

Thr202/Tyr204 expression were all decreased, while that of expression of STAT 5 and IFBP5 were increased after 6 months of treatment (9, 40-41). The alterations of gene profiles after 2 weeks of letrozole were also reported (11). In this study, there

were extensive changes in both up- and down-regulated gene profiling even after a short period of AI treatment. These data clearly indicated that ER-positive carcinoma cells developed extensive adaptive changes under estrogen depletion caused by AI treatment. Such adaptive changes are also reasonably considered partly to protect carcinoma cells from cellular apoptosis and render them to develop into phenotypic resistant strain. The recent demonstration of significant alterations of the enzymes estrogen sulfatase (STS) and 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1), other than aromatase being involved in intratumoral estrogen production, has also been considered in the spectrum of these adaptive cellular alterations responding to estrogen depletion (35). These adaptive changes may ultimately result in development of *de novo* resistance to AI. Normanno *et al.* also suggested that these adaptive changes are developed in a stepwise manner toward different types of endocrine therapies (42). In addition, these phenotypic changes have been usually considered to be derived from a series of protein interactions. It is therefore important to evaluate the changes of these proteins after exposure to endocrine therapy in the following two aspects. The first is to elucidate the mechanism of *de novo* resistance, which may provide novel therapeutic approaches. Secondly, the results may lead to an availability of potential predictors for subsequent adjuvant AI treatment, which is obviously of enormous help in determining the clinical management strategy.

To our knowledge, this is the first study reporting that the down-regulation of HSP-70 is significantly associated with treatment response of neoadjuvant AI in ER-positive postmenopausal breast cancer patients. Heat-shock proteins (HSPs) belong to a group of inducible proteins under various cellular stresses such as heat shock, chemotherapy and other anticancer therapies or other lethal conditions (43-44). HSPs are usually classified according to their molecular weights, such as HSP100, HSP90, HSP70, HSP60 and small HSPs. While the main cellular function of HSP is usually considered ATP-dependent protein chaperoning, HSP is also considered important in the process of post-translational protein-folding, keeping the proteins in correct configurations for their stability. This generally protects carcinoma cells from apoptosis [45]. Under stressful cellular conditions, elevated HSP-70 levels allow the cells to cope with increased levels of unfolded and denatured proteins. HSP-70 is therefore generally considered important in maintaining several house-keeping functions such as an import of proteins into cellular compartments; folding of proteins in the cytosol, endoplasmic reticulum and mitochondria; degradation of unstable proteins; dissolution of protein complexes; control of regulatory proteins; refolding of misfolded proteins; and translocation of precursor proteins into mitochondria (44). Thanner *et al.* demonstrated the correlations of HSP-70 expression with overall survival and survival after recurrence in node-negative breast cancer patients (46). Koshiyama *et al.* also reported that HSP-70 expression was related to either hormonal regulation of cell proliferation and/or down-regulation of sex steroid receptors in estrogen dependent human endometrium (47). Down-modulation of HSP-70 by anti-sense construct was also reported to have chemosensitizing and even cytotoxic properties *in vitro* (48-50). An inhibitor, ADD70 (AIF-derived decoy for HSP-70), was reported to demonstrate promising results in animal models for colon cancer and melanoma (51). In particular for breast cancer under the state of estrogen deprivation, ERs can be activated by non-ligand binding manner *via* cross-talk mechanisms by various signal transduction pathways, which at least includes Akt, MAPK and PI3K (42). One of the common sites for hyperphosphorylation by these kinases is Ser-118 loci of the ER (52). Since HSP-70 is closely related to Akt, the use of a novel HSP-70 inhibitor was reported to decrease Akt expression in a cell line study (53). This has explained the potential roles of HSP-70 in the cross-talking to ER under the stress of estrogen depletion.

In our present study, decreased levels of of HSP-70 in patients following the neoadjuvant therapy were associated with clinical and biological response to AI. It is unlikely that AI can directly down-regulate the expression of HSP-70 but those carcinoma cells unable to change the expression of HSP-70 to chaperone an increasing load of unfolded proteins and accommodate the need to stabilize the proteins involved in cross-talk mechanisms to ERs would have a greater chance of undergoing apoptosis; but further investigations are required to test this hypothesis.

## Disclosure/Conflict of interest

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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 $\geqq$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 $\geqq$ 10%) and 42 cases are ER negative (LI < 10%). Seventy-eight cases are PR positive (LI $\geqq$ 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

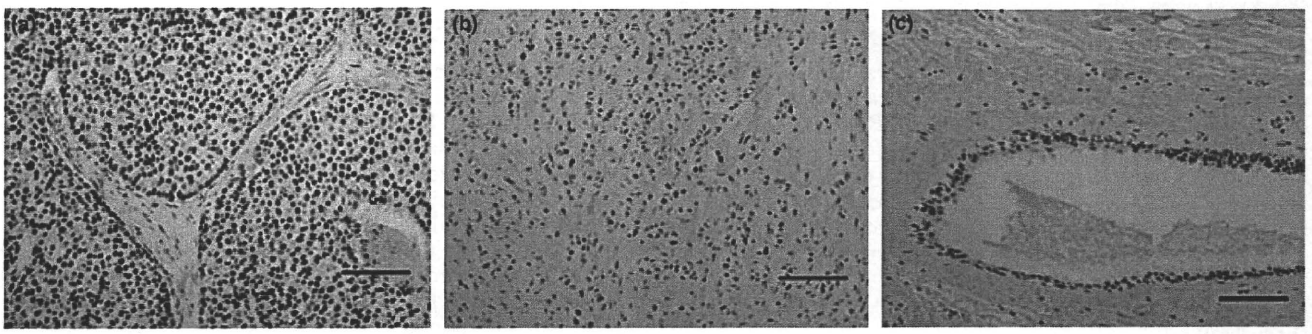


**Fig. 1.** Immunohistochemistry for Runx2. Runx2 immunoreactivity was detected in the nuclei of both carcinoma and stroma cells. (a) Case I with Stage III showed a labeling index (LI) = 97.0. (b) Case II with Stage II showed a LI = 37.0. (c) Normal mammary epithelial cells also include nucleus Runx2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

**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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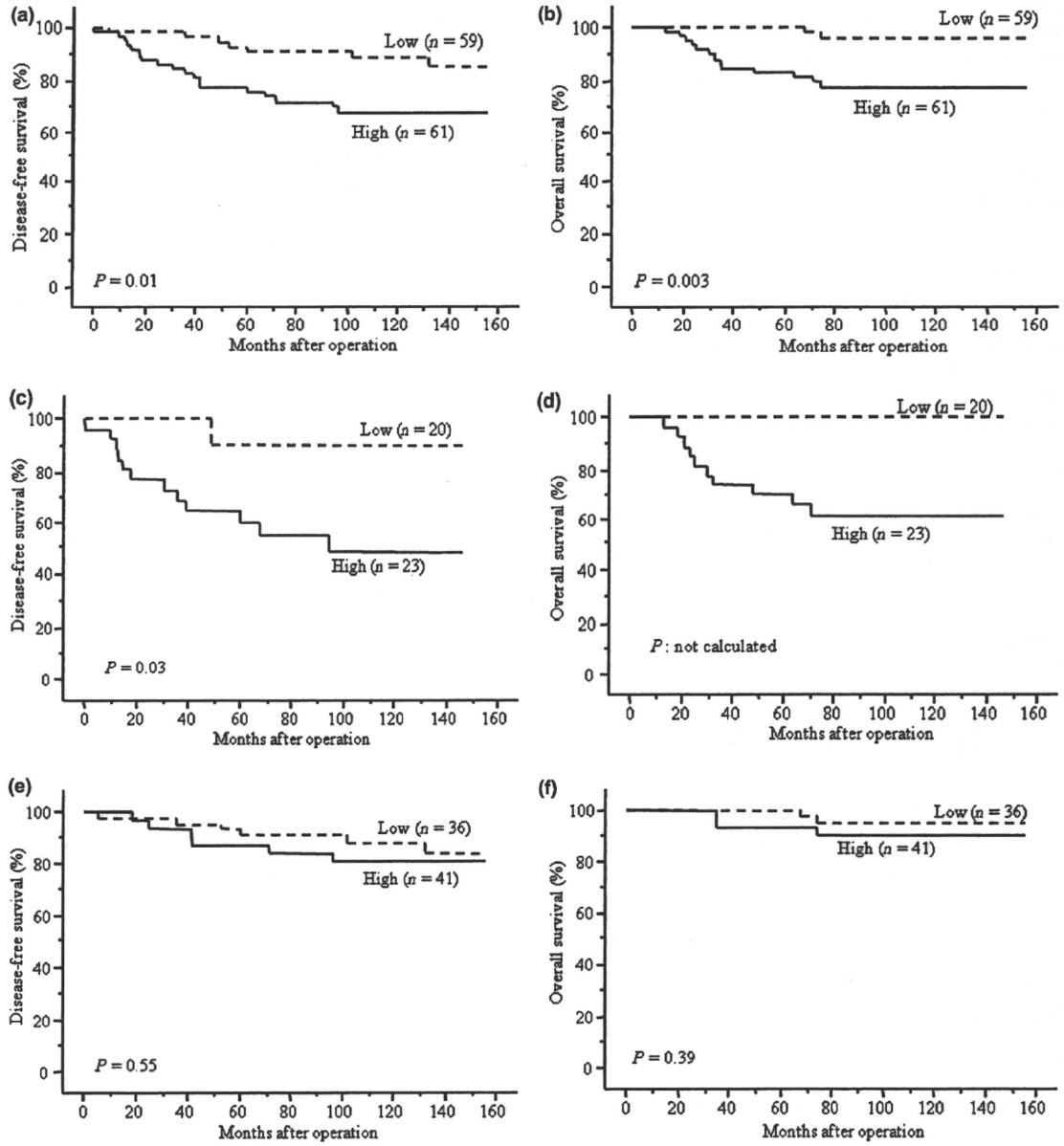


**Fig. 2.** Disease-free (a) and overall (b) survival of 120 cases associated with non-distal metastatic breast carcinoma according to nucleus Runx2 immunoreactivity (Kaplan–Meier method), respectively. Disease-free (c) and overall survival (d) of 77 estrogen receptor (ER)-positive breast carcinoma cases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

**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.



**Contents**



## 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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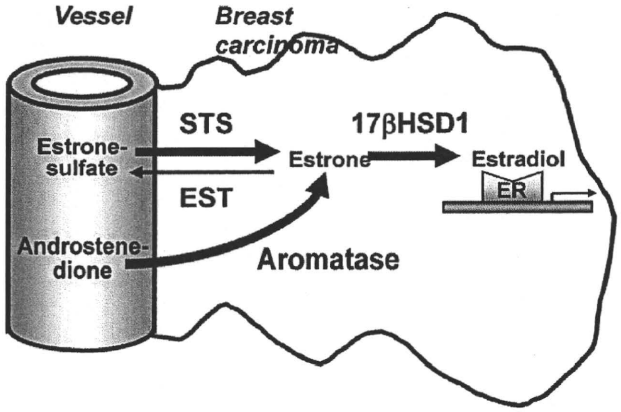


**Fig. 1.** Scheme representing *in situ* production of estrogens in the breast carcinoma tissue which is currently postulated. STS; steroid sulfatase, EST; estrogen sulfotransferase, 17βHSD1; 17β-hydroxysteroid dehydrogenase type 1, and ER; estrogen receptor.

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-

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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

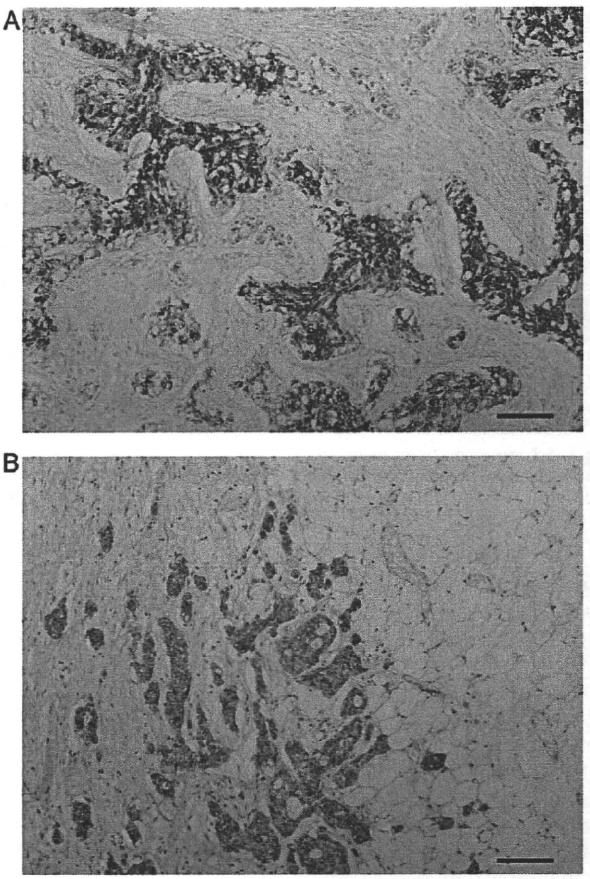


**Fig. 2.** STS (A) and EST (B) immunoreactivities in the breast carcinoma. Both immunoreactivities were detected in the cytoplasm of carcinoma cells. Bar = 100 μm, respectively.

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.

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-

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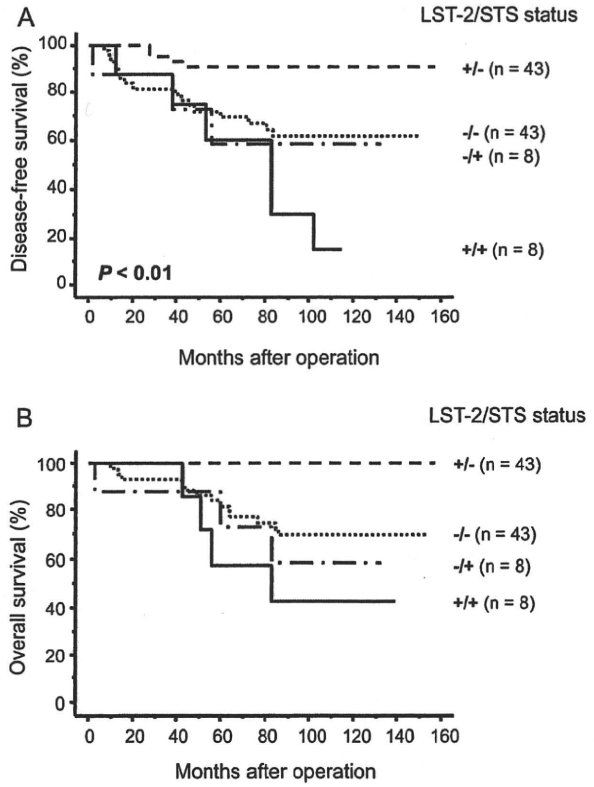


**Fig. 3.** Disease-free (A) and overall (B) survival of 102 patients with breast carcinoma according to LST-2/STS status (Kaplan–Meier method). LST-2/STS status was evaluated by immunohistochemistry. A+/+ group (*i.e.*, breast carcinoma positive for both LST-2 and STS) was significantly associated with 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-

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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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Review

# Inhibition of estrogen actions in human gynecological malignancies: New aspects of endocrine therapy for endometrial cancer and ovarian cancer

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ABSTRACT

Endometrial carcinoma and possibly ovarian carcinoma are considered "estrogen-dependent tumors" in human gynecological malignancies. In endometrial carcinoma, the enzymes responsible for intratumoral estrogen metabolism and biosynthesis are different from those in human breast carcinoma, although both of them are considered "estrogen-dependent malignancies". Specific and effective endocrine treatment of endometrial carcinoma should be explored, although progestin agents have been widely used for a long time. Aromatase inhibitors, the most effective endocrine agents of breast carcinoma, retinoids, metabolites of vitamin A, and synthetic peroxisome proliferator-activated receptor (PPAR) gamma ligands, used for the treatment of insulin resistance in type II diabetes mellitus, may be the important candidates for possible endocrine treatment of endometrial carcinoma.

In ovarian carcinoma, several clinical studies recently demonstrated that aromatase inhibitors had some therapeutic activity against recurrent ovarian carcinoma. However, at least at this juncture, further studies should be required to establish an aromatase inhibitor treatment as one form of endocrine therapy of ovarian carcinoma in future.



**Contents**



## 1. Endometrial carcinoma

### 1.1. *In situ* estrogen production and its regulation

#### 1.1.1. *Intracrinology*

Estrogen regulates a wide range of physiological responses in a variety of target tissues. It is well recognized that estrogen plays an important role in the development and progression of endometrial carcinoma. Endometrial carcinoma is one of the most common female pelvic malignancies in developed countries, and its

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incidence has recently increased (Jemal et al., 2004). Results of previous clinical, biological and epidemiological studies all demonstrated that excessive and/or prolonged exposure to estrogens not opposed by progesterone is considered to increase the risk of endometrial carcinoma, especially that of the endometrioid type (Thoma, 1984; Kelsey, 1982). However, the great majority of endometrial carcinomas arise during the postmenopausal period when ovaries ceased to be functional. Numerous studies examined the metabolism of serum estrogens in the patients diagnosed with endometrial carcinoma but there have been no consistent evidence of increased serum estrogen concentrations in postmenopausal women with endometrial carcinoma (Potischman et al., 1996; Sherman et al., 1997; Sasano and Harada, 1998; Lukanova et al., 2004).

Recently, a focus has been given to the importance of *in situ* estrogen metabolism, including synthesis and degradation, in the development and progression of various human estrogen-dependent neoplasms, including breast carcinoma. Results of several studies have demonstrated increased tissue estrogen content in human breasts, compared to serum and/or normal non-neoplastic tissues of the same patients (van Landeghem et al., 1985; Pasqualini and Chetrite, 1999; Chetrite et al., 2000). In these studies, the tissue concentrations of estrone (E1), estradiol (E2) and their sulfate metabolites were generally several times higher than those found in the plasma or in the area of the normal breast tissues of the same postmenopausal patients, despite markedly low levels of circulating estrogens. These findings all indicated specific intratumoral biosynthesis and accumulation of these hormones. However, there have been limited and inconsistent data regarding tissue estrogen concentrations in endometrial carcinoma tissues, in contrast to that for breast carcinoma (Bonney et al., 1986; Vermeulen-Meiners et al., 1986; Naitoh et al., 1989; Berstein et al., 2003). Berstein et al. (2003) examined 78 endometrial carcinomas and detected higher concentrations of E2 in cancer tissue specimens compared with macroscopically normal endometrium. Results from our previous studies were generally consistent with those of other investigations (Ito, 2005; Ito et al., 2006). We found that E2, estrone (E1), and testosterone levels in tumor tissues were several times higher than those measured in serum. These findings all indicate that intratumoral estrogen metabolism and synthesis are indeed important in the etiology and progression of endometrial carcinoma. Numerous studies have demonstrated that human endometrial carcinoma tissue contained the enzyme systems required for local biosynthesis of estrogen (Fig. 1). Among these enzymes, aromatase, 17β-hydroxysteroid dehydrogenases (17-HSDs) and steroid sulfatase (STS) are three principal enzymes involved in the formation of biologically active estrogen, estradiol. Estrogen-dependent neoplasms such as breast and endometrioid endometrial carcinoma, in which *in situ* conversions from serum androgens to biologically active estrogens occur, may be considered 'intracrine' tissues. However, the enzymes of 17-HSDs responsible for intratumoral estrogen production are markedly different between human breast and endometrial carcinomas.

### 1.1.2. Aromatase

Aromatase is an enzyme which catalyses the conversion of androgens, mainly androstenedione and testosterone, to E1 and E2, respectively (Bulun et al., 2005). Aromatase is a key enzyme in estrogen synthesis, and the levels of expression in endometrial cancer tissues have been demonstrated to be significantly higher than those in benign endometrial lesions. We previously demonstrated marked aromatase immunoreactivity and mRNA, mainly in the stromal cells or fibloblasts of endometrioid endometrial carcinoma, but not in normal or hyperplastic endometrium (Watanabe et al., 1995). Aromatase expression was significant, both at the protein and mRNA levels, at the site of frank invasion in endometrial

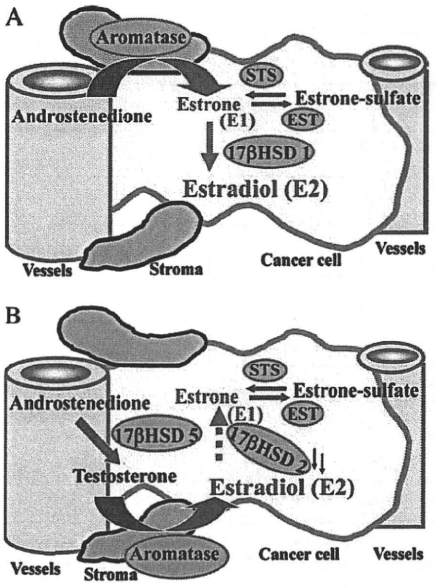


**Fig. 1.** Schema of intratumoral estrogens metabolism and synthesis in endometrial and breast carcinoma. (A) Schema of intratumoral estrogens metabolism and synthesis in breast carcinoma. (B) Schema intratumoral estrogens metabolism and synthesis in endometrial carcinoma. Androgens as substrate are supplied from the circulation. Androstenedione and testosterone are both converted into E1 and E2 by aromatase mainly in stromal cells, respectively. 17β-HSD types 1 and 2 catalyze the reversible conversion of E 1 and E2. The local regulation of estrogen activity is quite different between endometrial carcinoma and breast carcinoma. In breast carcinoma, E1 is produced by aromatase and STS. Then, 17β-HSD type 1 converts E1 to E2. 17β-HSD type 1 plays an important role in the regulation of high E2 levels in breast carcinoma tissues. However, 17β-HSD type 1 is not detectable and 17β-HSD type 2 and 5 are essential for the maintenance of E2 concentrations in endometrial carcinoma tissues. Testosterone is produced by 17β-HSD type 5 (conversion mainly from androstenedione to testosterone). In addition, E2 is produced by aromatase (conversion mainly from testosterone to E2). On the other hand, 17β-HSD type 2 expression is decreased through normal endometrium (secretory phase), hyperplasia and finally carcinoma accordingly.

carcinoma, suggesting an induction of aromatase expression by tumor–stromal interactions. In addition, a significant correlation was detected between aromatase expression in stromal cells and poor prognosis in patients with endometrial carcinoma (Segawa et al., 2005). This positive correlation indicates that local aromatase expression plays an important role in tumor progression through the formation of *in situ* estrogens.

It is very important to simulate *in vitro* stromal–carcinoma cell interactions when studying aromatase expression *in vitro*. However, the effects of tumor–stromal interactions on local estrogen biosynthesis in endometrial carcinomas have remained largely unknown. We therefore examined whether estrogen biosynthesis in the tumor microenvironment promotes endometrial carcinoma or not. In order to examine the contribution of stromal cells to estrogen signaling pathways in endometrial carcinoma, we used reporter cells stably transfected with the estrogen response element (ERE) fused to the destabilized green fluorescent protein (GFP) gene (Matsumoto et al., 2008). In this system, the endometrial cancer stromal cells from several patients activated the ERE of carcinoma cells to a variable extent. The GFP expression levels increased when testosterone, a substrate for aromatase, was added to the system. These effects were variably inhibited by aromatase inhibitors, although the response to aromatase inhibitors varied among the patients examined. These results all suggest that GFP expression is driven by estrogen synthesized by aromatase in the endometrial carcinoma stromal cells. In addition, in order to further confirm the local biosynthesis of estrogens and tumor–stromal interactions on aromatase activity, the endometrial carcinoma cell lines

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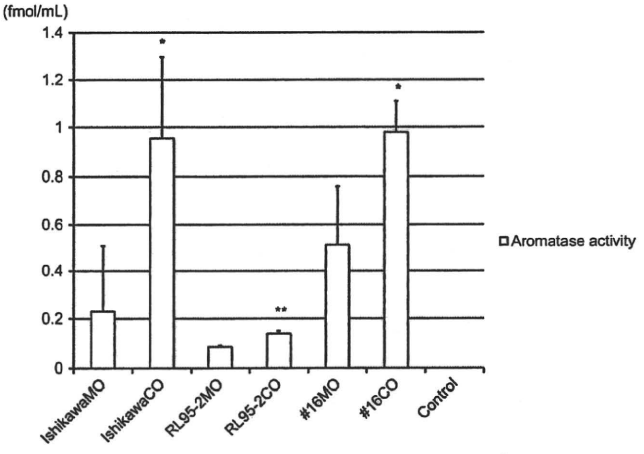


**Fig. 2.** Aromatase activity in IshikawaCO and RL95-2CO with #16 stromal cells was significantly higher than that in IshikawaMO and RL95-2MO. Aromatase assay was done for IshikawaMO, IshikawaCO, RL95-2MO, RL95-2CO, #16MO, and #16CO using 6a-methylandrost-4-ene-3,17-dione. The activity was measured by LC–MS/MS method. Mean ± SE. +, $P<0.01$ versus RL95-2MO; *, $P<0.05$ versus IshikawaMO and versus #16MO. CO; coculture, MO; monoculture, LC–MS/MS; liquid chromatography–tandem mass spectrometry.
Reproduced with permission from Takahashi-Shiga et al. (2009).

(Ishikawa and RL95-2) were co-cultured with stromal cells isolated from endometrial carcinomas, and aromatization activity was measured using liquid chromatography–tandem mass spectrometry (Takahashi-Shiga et al., 2009). We also examined the effects of aromatase inhibitors on cell proliferation. Aromatase activity was significantly higher in co-cultures with Ishikawa and RL95-2 than in each mono-culture, respectively. Cell proliferation was significantly inhibited in Ishikawa and RL95-2 cell cultures treated with aromatase inhibitors compared with control cultures. These *in vitro* findings in co-culture studies did confirm the importance of carcinoma-stromal cells interaction in the process of induction of aromatase in endometrial carcinoma tissues (Fig. 2).

Results of those *in vitro* and *in vivo* studies all demonstrated that aromatase is a key enzyme in the synthesis of estrogen in endometrial carcinoma as well as breast carcinoma.

*1.1.3. 17β-Hydroxysteroid dehydrogenase (17-HSD)*

*1.1.3.1. 17-HSD type 1 and 2.* The 17β-hydroxysteroid dehydrogenases (17-HSDs) catalyzes an interconversion of estrogens or androgens (Luu-The et al., 2001). Among the enzymes, 17-HSD types 1 and 2 catalyze the reversible interconversion of E1 and E2. Type 1 17-HSD catalyzes the 17β-reduction of biologically inactive E1 to E2 (Luu The et al., 1989; Gast et al., 1989), whereas the type 2 isozyme preferentially catalyzes the oxidation of E2 to E1 (Wu et al., 1993). Both type 1 and type 2 17-HSDs regulate tissue levels of E2 and modulate estrogenic actions in estrogen target tissues, such as the breast and endometrium (Sasano et al., 2000). Oxidative 17-HSD activity is the preferential reaction in normal human breast tissues, but the reductive 17-HSD pathway predominates in breast carcinomas. 17-HSD type 1 mRNA levels and intratumoral E2/E1 ratios were significantly higher in postmenopausal compared with premenopausal breast carcinomas (Miyoshi et al., 2001). 17-HSD type 1 immunoreactivity was detected in carcinoma cells in approximately 60% of breast carcinoma tissues, whereas 17-HSD type 2 was not expressed at all (Suzuki et al., 2000). Therefore, type 1 17-HSD is considered responsible for regulating the processes leading to the accumulation of E2 in human breast carcinomas.

In contrast, 17-HSD type 1 immunoreactivity was not detected in any of the cases of normal endometrium, endometrial hyperplasia and endometrioid endometrial carcinoma (Ito et al., 2001; Utsunomiya et al., 2001). 17-HSD type 1 mRNA expression and enzymatic activity were also absent in all carcinoma cases. In normal endometria, 17-HSD type 2 immunoreactive protein was detected only in the cytoplasm of glandular cells in the secretory phase. 17-HSD type 2 mRNA was also markedly expressed in the endometrial glandular epithelial cells of the luteal phase, but 17-HSD type 1 mRNA was not detected in any of the phases of the examined endometrium (Casey et al., 1994). 17-HSD type 2 immunoreactivity was detected in 75% and 37% of endometrial hyperplasia and endometrioid endometrial carcinoma cases, respectively. 17-HSD type 2 expression was decreased from normal endometrium (secretory phase) to hyperplasia and finally carcinoma (Utsunomiya et al., 2001). In addition, there was a statistically significant inversed correlation between the intratumoral E2 concentration and the level of 17-HSD type 2 mRNA in endometrial carcinoma (Ito et al., 2006). These results all suggest that type 2 17-HSD contributes to the regulation of "intratissue" estrogen levels in normal endometrium and that disruption of the regulatory mechanisms of intratissue estrogen levels may be related to the development and progression of endometrial disorders.

*1.1.3.2. 17-HSD type 5.* 17-HSD type 1 expression is negligible in human endometrioid endometrial carcinoma tissues and intratumoral E2 concentration may be maintained primarily by aromatization of testosterone in endometrial carcinoma. 17-HSD type 5, which catalyzes the reduction of androstenedione to testosterone, is a member of the aldo-keto reductase (AKR) superfamily, and is formally termed AKR1C3 (Dufort et al., 1999). This enzyme is expressed in various peripheral tissues, liver, prostate, and ovary, and has been also detected in prostate and breast carcinoma tissues (Luu-The et al., 2001; Suzuki et al., 2001; Vihko et al., 2004).

17-HSD type 5 immunoreactivity was reported to be present in glandular cells of endometrium, but not in stromal cells (Pelletier et al., 1999; Ito et al., 2006). In normal human endometrium, 17-HSD type 5 immunoreactivity was detected in 19% and 25% of proliferative and secretory phase endometria, respectively. However, 17-HSD type 5 immunoreactivity was detected in 50% and 69% of the cases of endometrial hyperplasia and endometrioid endometrial carcinoma, respectively. 17-HSD type 5 expression was increased significantly throughout normal endometrium, hyperplasia and finally carcinoma (Ito et al., 2006). In addition, there was a statistically significant inversed correlation between intratumoral testosterone concentration and aromatase mRNA level in endometrial carcinoma. Testosterone produced by 17-HSD type 5 in the tumor tissue may be finally aromatized to E2 by aromatase, which is also overexpressed in human endometrial cancer tissues. Although limited information is currently available on 17-HSD type 5 expression in endometrial malignancy, results of our studies did suggest that 17-HSD type 5 is one of the key enzymes in the local regulation of estrogen concentrations in endometrial malignancy.

*1.1.4. Steroid sulfatase (STS) and estrogen sulfotransferase (EST)*

Estrone sulfate (E1S), which is a biologically inactive form of estrogen, exhibits a relatively long half-life in the peripheral blood, and the levels of E1S are 5–10 times higher than those of unconjugated estrogens, such as E1 and E2, during the menstrual cycle and in postmenopausal women (Pasqualini, 2004). STS hydrolyzes circulating E1S to E1, whereas EST (estrogen sulfotransferase) sulfonates E1 to E1S. It was recently suggested that *in situ* estrogen activity in breast carcinoma may be primarily regulated by the status of intratumoral STS and EST (Suzuki et al., 2003). Therefore, the balance between the levels of intratumoral STS and EST may also play an important role in the regulation of *in situ* estrogen levels in estrogen-dependent neoplasms.

In normal endometrium, immunoreactivity to STS was not detected but that to EST was evident during the secretory phase of

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the cycle. Both STS and EST immunoreactivity have been detected in 86% and 29% of endometrial carcinomas cases, respectively. Their immunoreactivity was significantly correlated with those of enzymatic activity and mRNA level. In addition, the STS/EST ratio was associated significantly with poor prognosis in endometrial carcinoma patients (Utsunomiya et al., 2004). Similar results were also reported such as that STS/EST ratio of the mRNA level was increased in endometrial carcinoma tissues compared to adjacent normal endometrium (Smuc et al., 2006). Therefore, increased STS and decreased EST expression may contribute to regulation of *in situ* estrogen actions in human endometrial carcinomas, as similar to those in breast carcinomas. Recently, in a hormone-dependent endometrial xenograft model in ovariectomized nude mice, STS inhibitor was reported to be effective in inhibiting endometrial cancer growth (Foster et al., 2008). STS inhibitor may be one of the important candidates as a new endocrine-related agent in endometrial carcinomas as well as breast. However further clarification including pilot clinical studies should be necessary.

### 1.2. Endocrine therapy of endometrial carcinoma

#### 1.2.1. Progestin therapy

The physiological roles of progesterone in the regulation of the glandular epithelium of the endometrium are, in general, considered to antagonize estrogen-mediated cell proliferation and to induce cellular differentiation (Graham and Clarke, 1997). Progestins have been clinically demonstrated to provide some protection against the stimulatory effects of estrogenic agents. For example, hormone replacement therapy using combinations of estrogen and progestin yields a lower risk of endometrial carcinoma, despite an increment in the incidence of breast carcinoma (Beresford et al., 1997; Shumaker et al., 2003).

Accordingly, progestin agents have been extensively used for a long time in patients especially with advanced or recurrent endometrial carcinoma. In the Gynecologic Oncology Group (GOG), women with advanced or recurrent endometrial carcinoma were randomized to either low-dose (200 mg/day) or high-dose (1000 mg/day) medroxyprogesterone acetate (MPA), with response rate of 25% and 15%, respectively (Thigpen et al., 1999). There were no additional benefits to the dose greater than 200 mg/day, and the patients receiving the low-dose displayed a trend towards longer progression-free and overall survivals. This study concluded that oral MPA was active against advanced or recurrent endometrial carcinoma and response to progestin therapy was more frequent among patients with a well-differentiated histology and positive progesterone receptor status.

The standard initial treatment for endometrial carcinoma is obviously staging laparotomy with hysterectomy and bilateral salpingo-oophorectomy, which deprives these patients of any potential for fertility. Therefore, a more conservative medical treatment may be considered in young patients who may happen to wish to preserve their fertility. Approximately 3–5% of patients with these neoplasms are under age of 40, some of whom have been treated with progestin alone as a primary endocrine therapy for both atypical endometrial hyperplasia and early endometrioid endometrial adenocarcinoma. This approach has been supported by several reports in patients desiring to maintain fertility, despite the fact that the subsequent success rate of pregnancy is not necessarily high (Kaku et al., 2001; Shiozawa and Konishi, 2006). Some case reports have revealed that synthesized progestins such as MPA are effective as a conservative treatment of atypical hyperplasia and well differentiated endometrioid endometrial carcinoma. The initial response rate of MPA ranged from 55% to 100% for endometrial carcinoma and 70% to 100% for atypical hyperplasia (Kaku et al., 2001; Shiozawa and Konishi, 2006). In a recent multi-center prospective study, Ushijima et al. (2007) reported the results of 28 cases of endometrioid endometrial carcinoma at presumed stage IA, although it is impossible to verify, and 17 cases of atypical endometrial hyperplasia. Complete response was found in 55% of endometrial carcinoma cases and 82% of atypical hyperplasia cases. During the 3-year follow-up period, 12 pregnancies and seven normal deliveries were achieved after MPA therapy, although 14 recurrences were indeed detected in 30 patients (47%) between 7 and 36 months. Patients should be informed of these greater risks and absolute limitations of this conservative and controversial treatment.

Endometrial carcinoma, especially of the well-differentiated endometrioid type, and atypical hyperplasia often express PR, and their growth is suppressed by progestin. In general, the effect of progestin is considered to be mediated through PR, because the response rate to progestin therapy in PR-positive carcinoma was higher (75%) compared with PR-negative tumors (7%) (Kauppila, 1989). We previously demonstrated that the *in situ* abundance of 17-HSD type 2, which catalyzes the conversion of E2 to E1, and PR, especially PRB, can predict the responsiveness of patients with endometrioid endometrial carcinoma to progestin treatment (Utsunomiya et al., 2003). We also demonstrated that 17-HSD type 2 was only detected in the cytoplasm of the glandular cells during the secretory phase, but not in the proliferative phase endometrium (Utsunomiya et al., 2001). In addition, progestin stimulates the expression of 17-HSD type 2 in epithelial cells of human endometrial tissue (Yang et al., 2001). Progestin may exert a potent anti-estrogenic effect in the endometrium by inducing 17-HSD type 2 and thereby promoting the regression of endometrial proliferative disease.

#### 1.2.2. Aromatase inhibitor

Aromatase inhibitors have become the gold standard for endocrine treatments in the postmenopausal patients with estrogen-dependent breast carcinoma (Bulun et al., 2005). Though intratumoral aromatase activity is more frequently detected in endometrial endometrioid carcinoma than in breast carcinoma, there remain several controversies as to whether or not aromatase inhibitors are effective in patients with endometrial carcinoma. (Sasano et al., 1999; Rose et al., 2000; Berstein et al., 2005). We previously examined the biological changes in endometrial carcinoma tissues before and after aromatase inhibitor treatment. Five of 15 human endometrial carcinoma demonstrated decreased [$^3$H] thymidine uptake or Ki-67 labeling following aromatase inhibitor treatment (Sasano et al., 1999). Berstein et al. (2005) reported similar results. In our recent study of the local biosynthesis of estrogens and tumor–stromal interactions on aromatase activity, the endometrial carcinoma cell lines were co-cultured with stromal cells isolated from endometrial carcinomas. Cell proliferation was significantly inhibited in endometrial cancer cell cultures treated with aromatase inhibitors compared with control cultures (Takahashi-Shiga et al., 2009). These *in vitro* findings all suggest the effectiveness of aromatase inhibitors in endometrial carcinoma tissues.

However, the therapeutic value of aromatase inhibitor is still not clear at the moment. A GOG study was not able to demonstrate distinct clinical efficacy with aromatase inhibitor treatment. Partial responses were detected in 9% of 23 unselected patients with recurrent or persistent endometrial carcinoma, most of whom had poorly differentiated tumors (Rose et al., 2000). A multi-center phase II trial for letrozol was conducted in 32 recurrent or advanced endometrial carcinomas in postmenopausal women, and only one of 28 (4%) case had a complete response, two (7%) were associated with partial responses, and 11 out of 28 (39%) patients had a stable disease for a median duration of 7 months (Ma et al., 2004). Therefore, the roles of aromatase inhibitors in well-differentiated hormone-receptor-positive or hormone-sensitive endometrial car-

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