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Expression level of enzymes related to *in situ* estrogen synthesis and clinicopathological parameters in breast cancer patients

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ABSTRACT

In order to evaluate the importance of estrogen production in tumor and surrounding tissues, we measured mRNA expression levels of 5 enzymes participating to estrogen synthesis *in situ* and 4 breast cancer-related proteins in 27 pairs of tumor and non-malignant tissues. Steroid sulfatase (STS) mRNA was more frequently detected in tumor tissues rather than in their non-malignant counterparts. Estrogen sulfotransferase (EST) was constantly expressed with high level not only in tumor tissues but also in their surrounding non-malignant counterparts. In contrast, mRNA expression levels of aromatase, and 17β-hydroxysteroid dehydrogenase type I and II were relatively low and detected only in small proportion of the patients. We also measured the mRNA expression levels of the same nine genes in tumor tissues of 197 breast cancer patients, and analyzed relationship between the mRNA expression level and the clinicopathological parameters. The mRNA expression levels of STS, aromatase and erbB2 in tumor tissues increased as breast cancer progressed. The tumoral mRNA expression levels of STS, estrogen receptor β, and erbB2 in patients with recurrence were higher than those in patients without recurrence. Upregulation of STS expression plays an important role in tumor progression of human breast cancer and is considered to be responsible for estrogen production in tumor and surrounding tissues.

1. Introduction

Intratumoral metabolism and synthesis of estrogens as a result of the interactions of various enzymes are considered to play very important roles in the pathogenesis and development of hormone-dependent breast carcinoma [1–3]. Among these enzymes, intratumoral aromatase plays an important role converting serum androgens to estrogens *in situ*, especially in postmenopausal patients with breast cancer [4,5]. However, other enzymes such as the 17β-hydroxysteroid dehydrogenase (17β-HSD) isozymes, steroid sulfatase (STS) and estrogen sulfotransferase (EST) also play pivotal roles in intratumoral estrogen production [6,7]. 17β-HSD type I and II catalyze the interconversion of estrone (E1) and estradiol (E2). STS hydrolyzes estrone sulfate (E1S) to E1, whereas EST sulfonates E1 to E1S.

The factors that are generally known to have prognostic value in breast cancer include clinical stage, numbers of lymph node metastasis (*n*), which are the most significant predictors of outcome, and estrogen receptor (ER) levels, which serve as an indicator of response to hormonal therapy [8,9]. Recent studies have focused on intratumoral mRNA expression of enzymes involved in estrogen synthesis and discussed the correlation to clinicopathological parameters in separate reports [10–13]. However, it is difficult to see how these genes are expressed in concert. Yoshimura et al. have reported mRNA expressions of a numbers of genes in the estradiol pathway (i.e., aromatase, STS, 17β-HSD, EST), as well as ER and those involved ER signaling (i.e., Cyclin D1 and erbB2) in 155 breast cancer patients admitted in Norway, which have been measured simultaneously by fluorimetric quantitation of reverse transcriptase (RT)-PCR [14].

In the present study, we further explored such analysis using 197 tumor tissue samples obtained from Japanese breast cancer patients. In addition, we compared expression levels of those genes in breast cancer tissues with those in non-malignant breast tissues from 27 patients to investigate the role of each molecule in tumor environment.

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Table 1
Conditions of RT-PCR reaction.

Target	Primer sequence (top: RT/middle: PCR-forward/low: PCR-reverse)	Product size (bp)	Standard (amol/reaction)		PCR cycles (bp)
STS	5′-AGGGTCTGGGTGTGTCTGTC-3′ 5′F-ACTGCAACGCCTACTTAAATG-3′ 5′-AGGGTCTGGGTGTGTCTGTC-3′	287	0.2	355	24
EST	5′-CTTCATCTCTTGGAACGAAG-3′ 5′F-GTGGAAAAGTGCAAAGAAGATG-3′ 5′-CCTCTTTCAGGTCTTCGTAG-3′	397	5	397	22
Aromatase	5′-AACCACGATAGCACTTTCGT-3′ 5′F-TACTACAACCGGGTATATGG-3′ 5′-TGTTAGAGGTGTCCAGCATG-3′	378	0.005	399	33
17β-HSD I	5′-CGAAAGACTTGCTTGCTGTG-3′ 5′F-GGACGTGCTGGTGTGTAAC-3′ 5′-CTCTGGGCTGCCCAACAC-3′	352	0.05–0.1	352	28
17β-HSD II	5′-CATGCTGCTGACATTCACCA-3′ 5′F-GGTGTCATGCTTCCTCATGT-3′ 5′-CTTTGTGACCTCCACAGTTC-3′	418	0.05–0.1	418	28
ERα	5′-GCCTTTGTTACTCATGTGCC-3′ 5′F-CTGATGATTGGTCTCGTCTG-3′ 5′-GTGTCTGTGATCTTGTCCAG-3′	295	0.05–0.1	295	28
ERβ	5′-CAGGAGCATCAGGAGGTTA-3′ 5′F-CCGACAAGGAGTTGGTACA-3′ 5′-TCAGCAAGTGAGCCAGCTTC-3′	387	0.05–0.1	387	28
Cyclin D1	5′-GTCACACTTGATCACTCTGG-3′ 5′F-CCTACTTCAAATGTGTGCAG-3′ 5′-CCAGGTTCCACTTGAGCTTG-3′	328	0.05–0.1	328	27
ErbB2	5′-GTCAATCATCCAACATTTGACC-3′ 5′F-AGCTGGTGACACAGCTTATG-3′ 5′-CCAAAAGTCATCAGCTCCCA-3′	372	0.05–0.1	372	28
β-Actin	5′-ACGTCACACTTCATGATGGA-3′ 5′F-GTGATGGACTCCGGTGACGG-3′ 5′-CAGCGGAACCGCTCATTGC-3	317	0.05–0.5	317	25 or 27

2. Materials and methods

2.1. Tissue samples

Frozen tissue samples obtained from patients who underwent resection of breast cancer at National Shikoku Cancer Center were used. They were stored at −80 °C until use. The 27 patients in whom tumor tissues was compared with its non-malignant counterpart had a mean age of 53.9 ± 9.9 years (median, 55 years; range, 29–74 years), with a mean follow-up period of 632 ± 299 days (median, 705 days) as of March 31, 1999. The 197 patients analyzed for the mRNA expression levels of each analyte in breast tumor tissues had a mean age of 52.9 ± 10.9 years (median, 51 years; range, 29–84 years), with a mean follow-up period of 741 ± 493 days (median, 756 days). Research protocols for this study were approved by the Ethics Committee at Shikoku Cancer Center.

2.2. RNA preparation and assay

The frozen tissue samples were placed in a glass tube for homogenization and immediately homogenized (DIGITAL HOMOGENIZER, Iuchishoei-do, Tokyo, Japan) with 1 mL of TRIZOL (Invitrogen, Carlsbad, CA). It was allowed to stand at room temperature for 10 min and stored at −80 °C until RNA extraction. The homogenate was thawed at room temperature, followed by the addition of 0.2 mL of chloroform (Wako Pure Chemical Industries, Osaka, Japan). The mixture was vortexed for 20 s, allowed to stand at room temperature for 5 min, and centrifuged at 4 °C for 15 min. A 0.5-mL aliquot of the supernatant (aqueous layer) was mixed with 0.47 mL of isopropanol and allowed to stand at room temperature for 10 min, followed by centrifugation at 4 °C for 15 min. The resulting RNA pellet was washed with 75% cold ethanol and dried under reduced pressure. The residue was dissolved in $T_{10}E_{0.2}$ (10 mM Tris–HCl–0.2 mM EDTA, pH 8.0), and a 1-μL aliquot of the resulting solution was retained for spectrophotometrical RNA assay. After the addition of 1 μL of Prime RNase Inhibitor (Eppendorf, Hamburg, Germany), the remaining portion was stored at −80 °C until RT reaction. RNA levels were assayed by determining OD_{260} (1OD = 40 μg/mL) in the solution diluted 10-fold with diethylpyrocarbonate-treated water (BECKMAN DU-600).

2.3. RT reaction

To 0.5 μg of RNA, the RT reaction mixture [50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3.5 mM $MgCl_2$, 15 mM DTT, 1.25 mM dNTP mix, 50 pmol RT primer, 0.2 amol STS internal standard or 0.005 amol aromatase internal standard, 30 units Prime RNase Inhibitor, and 100 units MMLV reverse transcriptase (Invitrogen)] was added to give a total volume of 20 μL. RNA was reverse transcribed at 47 °C for 45 min, followed by the addition of 5 units of RNase H (Takara Shuzo, Otsu, Japan). The mixture was incubated at 37 °C for 30 min for RNA degradation. The reaction was stopped by heating at 95 °C for 5 min and quenched, followed by the addition of 80 μL of water. The resulting cDNA solution was stored at −20 °C until PCR. Sequences of RT primer used were shown in Table 1.

2.4. PCR and mRNA assay

Conditions of RT-PCR reaction were shown in Table 1. To 10 μL of the cDNA solution, the PCR reaction mixture [10 mM Tris–HCl (pH 8.7), 45 mM KCl, 1.5 mM $MgCl_2$, 0.125 mM dNTP mix, 5 μg/mL gelatin, 20 pmol antisense primer, 6.25–12.5 pmol a fluorescent dye, FAM (PerkinElmer Co., Foster City, CA, USA)-labeled sense primer, 1.25 units Taq DNA polymerase (PerkinElmer)] was added to give a total volume of 40 μL. PCR was performed under the following conditions: 94 °C, 15 s → 55 °C, 30 s → 72 °C, 30 s (22–33 cycles) → 72 °C, 10 min. In the presence of a size standard (GENESCAN-1000 ROX; PerkinElmer), the reaction product was electrophoresed on 2% agarose gel and analyzed with a Gene Scanner 362 Fluorescent Fragment Analyzer (PerkinElmer). The mRNA expression levels of STS and aromatase were calculated from the ratio of the peak area of cDNA derived from each analyte to that of the internal standard at a known concentration by the internal standard method. Peak areas were determined with a fluorescence detector. For the other analytes, mRNA expression levels were calculated from the ratio of the peak area of cDNA derived from each analyte to that of the standard substance at a known concentration that had been subjected to PCR in a separate tube. For all analytes, mRNA expression levels per unit of β-actin, taken as a house keeping gene, were used for statistical analysis.

2.5. Statistical analysis

Statistical analysis was performed using SAS (ver. 6.12). The Mann–Whitney *U* test was used to compare median mRNA expression levels between two groups. For comparisons between three or more groups, the Kruskal–Wallis test was applied, followed by the Steel-Dwass test if the former test revealed significant differences ($p < 0.05$). Contingency table analysis was performed using the χ^2 test or the Fisher exact probability test.

Table 2
Messenger RNA levels of estrogen metabolizing enzymes (STS, EST, aromatase, and 17β-HSD I and II) in breast cancer tissues and non-malignant breast tissues from 27 breast cancer patients.

Tissue	mRNA levels (top: mean ± SD[a], middle: range[a], low: number of detected samples)				
	STS	EST	Aromatase	17β-HSD I	17β-HSD II
Breast cancer	0.085 ± 0.145 0–0.504 18 (66.7%)	114 ± 92 0–357 25 (92.6%)	0.0009 ± 0.0019 0–0.0078 12 (44.4%)	0.028 ± 0.105 0–0.533 4 (14.8%)	0.210 ± 1.086 0–5.64 3 (11.1%)
Non-malignant breast	0.148 ± 0.290 0–1.168 10 (37.0%)	121 ± 205 0–947 20 (74.1%)	0.0029 ± 0.0074 0–0.0359 10 (37.0%)	0.013 ± 0.024 0–0.079 7 (25.9%)	0.002 ± 0.008 0–0.044 1 (3.7%)

[a] Unit, amol/amol β-actin.

3. Results

3.1. Messenger RNA expression in breast cancer and non-malignant tissues

Messenger RNA levels of 5 enzymes participating to estrogen synthesis (STS, EST, aromatase, and 17β-HSD type I and II) in breast cancer tissues and in non-malignant breast tissues from 27 breast cancer patients are shown in Table 2 and Fig. 1A–C. STS mRNA was detected 66.7% in the breast cancer tissues, while it was detected only 37.0% in the non-malignant breast tissues. However, no significant difference in the mean value was detected between breast cancer tissues (0.085 ± 0.145 amol/amol β-actin, mean ± SD) and non-malignant breast tissues (0.148 ± 0.290 amol/amol β-actin). EST mRNA was detected 92.6% in the breast cancer tissues and 74.1% in the non-malignant breast tissues. EST mRNA levels were relatively high, not only in breast cancer tissues (114 ± 92 amol/amol β-actin) but also in non-malignant breast tissues (121 ± 205 amol/amol β-actin). No significant difference was detected among them. Aromatase mRNA was almost equally detected in both tissues (breast cancer tissues: 44.4%; non-malignant breast tissues: 37.0%). Both HSD isoenzymes mRNA were typically few detected in both tissues (17β-HSD type I: 14.8% in the breast cancer tissues and 25.9% in the non-malignant breast tissues; 17β-HSD type II: 11.1% in the breast cancer tissues and 3.7% in the non-malignant breast tissues).

Messenger RNA levels of 4 breast cancer-related proteins (ERα, ERβ, Cyclin D1, and erbB2) in 27 pairs of tumor and non-malignant tissues are shown in Table 3 and Fig. 1D–G. ERα and erbB2 mRNA in breast cancer tissues were detected in more than 80%,

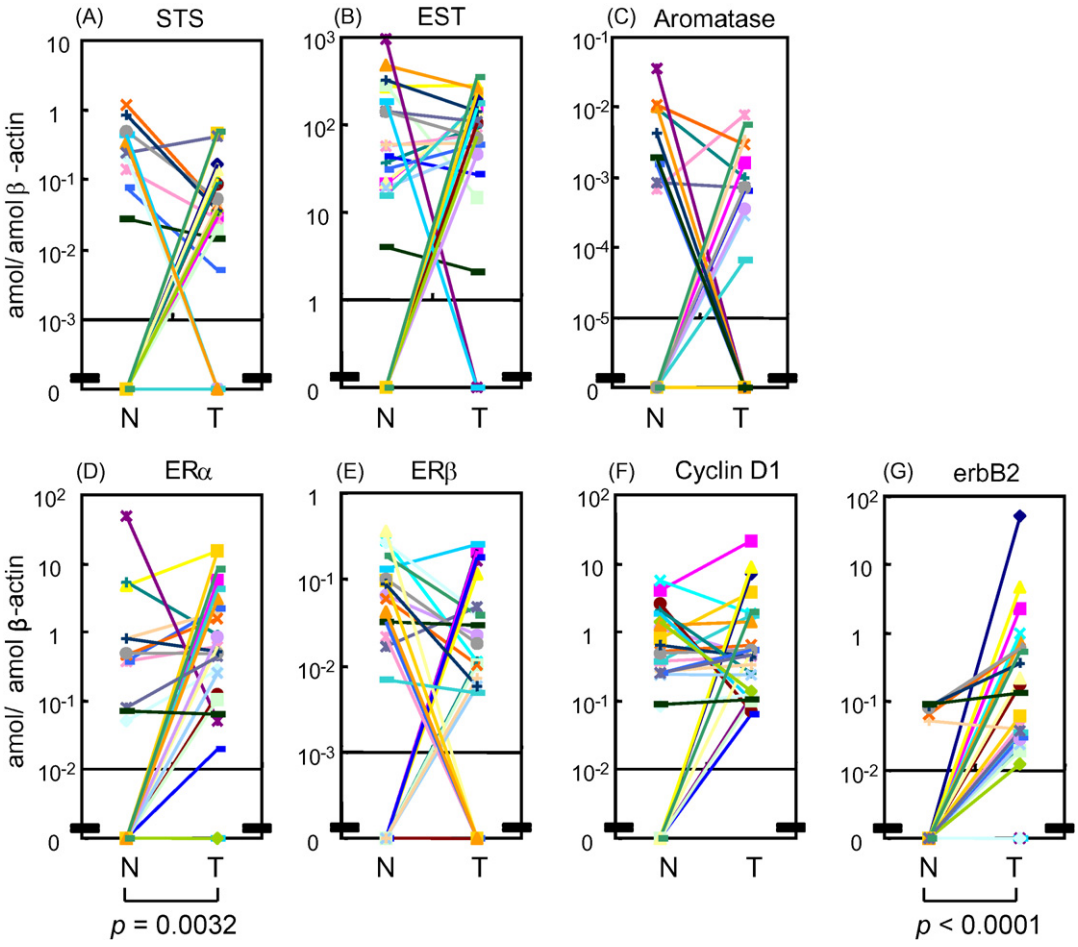

Fig. 1. Relationship between mRNA levels in breast cancer tissues and those in non-malignant breast tissues from 27 breast cancer patients. *p* values were calculated with Mann–Whitney *U* test. N: non-malignant breast tissues; T: breast cancer tissues. Zero represents the value to be lower than detectable range.

Table 3
Messenger RNA levels of breast cancer-related proteins (ERα, ERβ, Cyclin D1, and erbB2) in breast cancer tissues and non-malignant breast tissues from 27 breast cancer patients.

Tissue	mRNA levels (top: mean ± SD[a], middle: range[a], low: number of detected samples)			
	ERα	ERβ	Cyclin D1	ErbB2
Breast cancer	2.35 ± 4.19 0–15.4 24 (88.9%)	0.053 ± 0.080 0–0.261 20 (74.1%)	1.99 ± 4.41 0.062–21.4 27 (100%)	2.29 ± 9.63 0–50.3 23 (85.2%)
Non-malignant breast	2.27 ± 9.16 0–47.6 12 (44.4%)	0.070 ± 0.098 0–0.368 17 (63.0%)	0.905 ± 1.36 0–5.69 20 (74.1%)	0.014 ± 0.031 0–0.092 5 (18.5%)

[a] Unit, amol/amol β-actin.

Table 4
Messenger RNA levels of estrogen metabolizing enzymes (STS, EST, aromatase, and 17β-HSD I and II) and breast cancer-related proteins (ERα, ERβ, Cyclin D1, and erbB2) in the tumors of 197 breast cancer patients.

Transcript	Mean ± SD[a]	Median[a]	Range[a]	Number of detected samples
STS	0.034 ± 0.065	0.007	0–0.48	107 (54.3%)
EST	69.9 ± 78.0	45.1	0–592	185 (93.9%)
Aromatase	0.00074 ± 0.0036	0	0–0.0376	51 (25.9%)
17β-HSD I	0.014 ± 0.101	0	0–1.34	20 (10.1%)
17β-HSD II	0.013 ± 0.066	0	0–0.55	24 (12.2%)
ERα	1.46 ± 2.22	0.62	0–15.4	154 (78.2%)
ERβ	0.012 ± 0.028	0.001	0–0.26	102 (51.8%)
Cyclin D1	0.88 ± 1.31	0.37	0–8.67	184 (93.4%)
ErbB2	1.57 ± 4.05	0.35	0–25.3	169 (85.8%)

[a] Unit, amol/amol β-actin.

while those in non-malignant breast tissues were detected in less than half. Both ERα and erbB2 mRNA levels in breast cancer tissues were significantly higher than those in non-malignant breast tissues ($p = 0.0032$ and <0.0001, respectively). ERβ mRNA was equally detected in both tissues (breast cancer tissues: 74.1%; non-malignant breast tissues: 63.0%). Cyclin D1 mRNA was detected in all cancer tissues and in 74.1% of non-malignant breast tissues. No significant difference was detected between the mean value in breast cancer tissues (1.99 ± 4.41 amol/amol β-actin) and that in non-malignant breast tissues (0.905 ± 1.36 amol/amol β-actin).

3.2. Messenger RNA expression in 197 breast cancer patients

Messenger RNA levels of STS, EST, aromatase, 17β-HSD types I and II, ERα, ERβ, Cyclin D1, and erbB2 in tumor tissues of 197 breast cancer patients are shown in Table 4. STS mRNA was detected in about half of the patients. EST mRNA was detected in more than 90% of the patients, and aromatase mRNA was detected only in 25.9% of the patients. Messenger RNA levels of both HSD isoenzymes were very few detected (only in about 10% of the patients). ERα, ERβ, Cyclin D1 and erbB2 mRNA were detected in about 50–90% of the patients.

Correlations of expression levels of the various transcripts measured were analyzed using Pearson correlation (Table 5). Expression levels of STS mRNA correlated with those of EST ($r = 0.296$, $p < 0.01$), aromatase ($r = 0.205$, $p < 0.01$), ERα ($r = 0.240$, $p < 0.01$), Cyclin D1 ($r = 0.277$, $p < 0.01$) and erbB2 ($r = 0.238$, $p < 0.01$). Expression levels of EST mRNA correlated with those of ERα ($r = 0.508$, $p < 0.01$) and Cyclin D1 ($r = 0.451$, $p < 0.01$) mRNA. Expression levels of 17β-HSD type I mRNA correlated with those of Cyclin D1 ($r = 0.294$, $p < 0.01$) and erbB2 ($r = 0.448$, $p < 0.01$) mRNA. Expression levels of both ERα mRNA ($r = 0.541$, $p < 0.01$) and ERβ mRNA ($r = 0.147$, $p < 0.05$) also correlated with those of Cyclin D1 mRNA.

3.3. STS, EST and aromatase mRNA expressions and clinical and histopathological parameters

The levels of mRNA expression of genes which involved in estrogen synthesis, was then studied in relation to different clinical and histopathological parameters; age, tumor size, menopausal status, nodal status, clinical stage, ER status and recurrence. Clinical and histopathological parameters of the 197 breast cancer patients are shown in Table 6. Forty three patients (21.8%) and 131 patients (66.5%) were in clinical stage 1 and stage 2, and 21 patients (10.6%) and only 1 patient (0.5%) were in clinical stage 3 and stage

Table 5
Pearson correlation of the expression levels of mRNA coding for estrogen metabolizing enzymes and breast cancer-related proteins.

	STS	EST	Aromatase	17β-HSD I	17β-HSD II	ERα	ERβ	Cyclin D1	erbB2
STS	1	0.296**	0.205**	0.139	0.116	0.240**	0.123	0.277**	0.238**
EST		1	0.068	0.081	0.004	0.508**	0.026	0.451**	0.113
Aromatase			1	−0.006	0.064	−0.025	0.101	0.016	0.021
17β-HSD I				1	−0.026	−0.004	0.046	0.294**	0.448**
17β-HSD II					1	−0.095	−0.031	−0.002	0.126
ERα						1	−0.066	0.541**	−0.086
ERβ							1	0.147*	−0.007
Cyclin D1								1	0.115
erbB2									1

* $p < 0.05$.
** $p < 0.01$.

Table 6
Clinical and histopathological parameters of a set of 197 breast cancer patients.

Clinical and histopathological parameters		Number	Ratio (%)
Age (yr)	<50	90	45.7
	≥50	107	54.3
Menopausal status	Pre	101	51.3
	Post	84	42.6
	Post L[a]	10	5.1
	Unknown	2	1.0
Tumor size (mm)	≤20	45	22.8
	>20	152	77.2
Nodal status	Negative	160	81.2
	Positive	37	18.8
Stage	0	1	0.5
	1	43	21.8
	2	131	66.5
	3a	15	7.6
	3b	6	3.0
	4	1	0.5
ER status	Positive	114	57.9
	Negative	80	40.6
	Unknown	3	1.5
Adjuvant chemotherapy	No	110	55.8
	Yes	83	42.1
	Unknown	4	2.0
Adjuvant endocrine therapy	No	58	29.4
	Yes	136	69.0
	Unknown	3	1.5
Recurrence	No	189	95.9
	Yes	8	4.1

[a] Post L represents postmenopausal status induced by LHRH-agonist therapy.

4, respectively. Approximately 70% of patients received adjuvant endocrine therapies. Most of the patients (95.9%) had no recurrence at the time analyzed (follow-up period: 37–2680 days; median: 750 days).

Messenger RNA level of STS increased as the clinical stage advanced (Table 7, Fig. 2A). Messenger RNA levels of STS in nodal-positive patients were significantly higher than those in nodal-negative patients ($p < 0.05$) where only detected cases were analyzed. Messenger RNA levels of STS in patients with recurrence were also higher than those in patients without recurrence (Table 7). Messenger RNA levels of EST in patients with clinical stage 2 were significantly higher than those in patients with clinical stage 0 and 1 (Fig. 2B). Messenger RNA levels of aromatase in patients with clinical stage 3 and 4 were significantly higher than those in patients with clinical stage 2 ($p < 0.05$), where only samples showing positive values were selected for the analysis (Table 7).

3.4. ERα, ERβ, Cyclin D1 and erbB2 mRNA expressions and clinical and histopathological parameters

Relationships between the expression levels of ERα, ERβ, Cyclin D1 and erbB2, and clinical and histopathological parameters are shown in Table 8. Messenger RNA levels of ERα in ER-positive patients were significantly higher than those in ER-negative patients ($p < 0.05$), as ER protein was determined by ELISA for ERα. Messenger RNA levels of ERβ in patients with recurrence were significantly higher than those in patients without recurrence. No significant change in the mRNA levels of ERβ was shown by ER status. Messenger RNA levels of Cyclin D1 in patients with clinical stage 2 were significantly higher than those in patients with clinical stage 0 and 1 (Fig. 2C), and mRNA levels of Cyclin D1 in the ER-positive patients were significantly higher than those in the ER-negative patients ($p < 0.05$). Messenger RNA levels of erbB2 elevated as the clinical stage advanced (Fig. 2D), and were significantly higher in patients with recurrence ($p < 0.05$).

4. Discussion

First, we measured mRNA expression level of 5 enzymes participating to estrogen synthesis *in situ* and 4 breast cancer-

Table 7
Median values of mRNA expression of estrogen metabolizing enzymes (STS, EST, and aromatase) in relation to the clinical and histopathological parameters in the tumors of 197 breast cancer patients.

Clinical and histopathological parameters		STS		EST		Aromatase	
		All	*Detected* #	*All*	*Detected*	*All*	*Detected*
Age (yr)	<50	0.001 † (90)	0.032 (45)	51.6 (90)	54.6 (88)	0.00000 (90)	0.00056 (22)
	≥50	0.011 (107)	0.042 (62)	42.6 (107)	49.6 (97)	0.00000 (107)	0.00054 (29)
Menopausal status	Pre	0.002 (101)	0.034 (52)	49.0 (101)	49.5 (99)	0.00000 (101)	0.00073 (25)
	Post	0.010 (84)	0.042 (49)	43.8 (84)	54.6 (75)	0.00000 (84)	0.00043 (22)
Tumor size (mm)	≤20	0.000 (45)	0.037 (20)	38.9 (45)	43.4 (41)	0.00000 (45)	0.00068 (12)
	>20	0.008 (152)	0.038 (87)	49.3 (152)	54.9 (144)	0.00000 (152)	0.00046 (39)
Nodal status	Negative	0.006 (160)	0.033 (87) *)	45.2 (160)	53.6 (151)	0.00000 (160)	0.00054 (41)
	Positive	0.009 (37)	0.090 (20)	45.1 (37)	52.4 (34)	0.00000 (37)	0.00084 (10)
Stage	0&1	0.000 (44) *)	0.034 (16)	15.3 (44) **)	18.5 (38) **)	0.00000 (44)	0.00050 (9)
	2	0.011 (131)	0.037 (79)	58.5 (131)	60.9 (126)	0.00000 (131)	0.00037 (34) *)
	3&4	0.013 (22)	0.075 (12)	36.8 (22)	38.2 (21)	0.00000 (22)	0.00314 (8)
ER status	Positive	0.002 (114)	0.034 (59)	55.6 (114)	57.6 (108)	0.00000 (114)	0.00036 (30)
	Negative	0.015 (80)	0.042 (46)	41.7 (80)	44.6 (74)	0.00000 (80)	0.00099 (21)
Recurrence	No	0.006 (189)	0.037 (104) *)	45.1 (189)	52.1 (178)	0.00000 (189)	0.00060 (48)
	Yes	0.042 (8)	0.098 (5)	36.1 (8)	53.6 (7)	0.00000 (8)	0.00023 (3)

#Only samples showing positive values, †unit, amol/amol β-actin, *$p < 0.05$, **$p < 0.01$. Number in parenthesis shows number of patients.

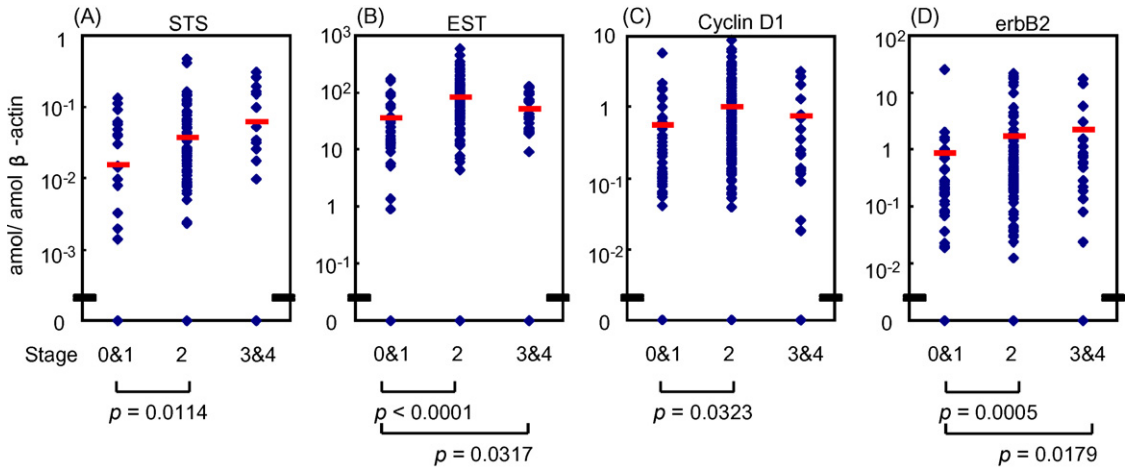

Fig. 2. Correlation between mRNA levels and clinical stage in the tumors of 197 breast cancer patients. *p* values were calculated with Steel-Dwass test followed Kruskal–Wallis test. *p* values derived from Kruskal–Wallis test were 0.011 (A, stage 0 and 1 vs. 2), <0.0001 and 0.0317 (B, stage 0 and 1 vs. 2 and stage 0 and 1 vs. 3 and 4, respectively), 0.0323 (C, stage 0 and 1 vs. 2), and 0.0005 and 0.0179 (D, stage 0 and 1 vs. 2 and stage 0 and 1 vs. 3 and 4, respectively).

Table 8
Median values of mRNA expression of breast cancer-related proteins (ERα, ERβ, Cyclin D1, and erbB2) in relation to the clinical and histopathological parameters in the tumors of 197 breast cancer patients.

Clinical and histopathological parameters		ERα		ERβ		Cyclin D1		erbB2	
		All	*Detected* #	*All*	*Detected*	*All*	*Detected*	*All*	*Detected*
Age (yr)	<50	0.62 † (90)	0.89 (75)	0.000 (90)	0.008 (41)	0.37 (90)	0.49 (84)	0.35 (90)	0.42 (84)
	≥50	0.61 (107)	1.06 (79)	0.004 (107)	0.012 (61)	0.37 (107)	0.43 (100)	0.32 (107)	0.52 (85)
Menopausal status	Pre	0.62 (101)	0.88 (85)	0.000 (101)	0.008 (48)	0.39 (101)	0.45 (96)	0.35 (101)	0.41(94)
	Post	0.75 (84)	1.49 (60)	0.003 (84)	0.011 (48)	0.37(84)	0.43 (78)	0.34 (84)	0.56(67)
Tumor size (mm)	≤20	0.59 (45)	0.83 (34)	0.005 (45)	0.012 (27)	0.35 (45)	0.37 (43)	0.29 (45)	0.37 (39)
	>20	0.67 (152)	1.00 (120)	0.000 (152)	0.009 (75)	0.38 (152)	0.46 (141)	0.37 (152)	0.48 (130)
Nodal status	Negative	0.75 (160)	1.03 (130)	0.001 (160)	0.010 (81)	0.39 (160)	0.45 (153)	0.35 (160)	0.46 (135)
	Positive	0.23 (37)	0.83 (24)	0.004 (37)	0.015 (21)	0.26 (37)	0.45 (31)	0.32 (37)	0.43 (34)
Stage	0&1	0.53 (44)	0.86 (33)	0.001 (44)	0.011 (23)	0.24 (44)	0.29 (41)	0.14 (44)	0.21(41)
	2	0.63 (131)	0.95 (105)	0.001 (131)	0.010 (68)	0.46 (131)	0.51 (123)	0.42 (131)	0.47 (117)
	3&4	0.91 (22)	1.65 (16)	0.002 (22)	0.008 (11)	0.24 (22)	0.30 (20)	0.59 (22)	0.68 (20)
ER status	Positive	0.92 (114)	1.03 (107)	0.001 (114)	0.010 (58)	0.49 (114)	0.52 (110)	0.30 (114)	0.44 (96)
	Negative	0.06 (80)	0.86 (45)	0.002 (80)	0.011 (43)	0.24 (80)	0.31 (71)	0.39 (80)	0.46 (70)
Recurrence	No	0.69 (189)	1.03 (148)	0.001 (189)	0.010 (96)	0.39 (189)	0.46 (176)	0.34 (189)	0.44 (161)
	Yes	0.29 (8)	0.43 (6)	0.018 (8)	0.027 (6)	0.25 (8)	0.25 (8)	1.69 (8)	1.69 (8)

#Only samples showing positive values, †unit, amol/amol β-actin, $^{*}p < 0.05$, $^{**}p < 0.01$. Number in parenthesis shows number of patients.

related proteins in 27 pairs of tumor and non-malignant tissues samples.

The present study showed that STS mRNA was more frequently detected in tumor tissue samples rather than in their non-malignant counterparts in 12 out of the 27 patients. However the difference in the average of STS mRNA among malignant and non-malignant tissues was not statistically significant because of large variation among measured samples. Utsumi et al. [15] has reported that mRNA levels of STS in tumor tissues were higher than those in non-malignant tissues in 33 out of the 38 patients. The average of STS mRNA in malignant tissues was more than twice of that in non-malignant counterparts and the difference between them was statistically significant. Enzymatic activity for STS in breast cancer tissues has been reported to be higher than that in non-malignant tissues [16,17]. Results from our present study and previous findings suggest that STS is involved in the *in situ* activation of E1 from E1S, thereby contributing to the increment of estrogenic action in human breast cancer tissues.

In this study, the mean mRNA level of EST was much higher than that of the other four enzymes involving estrogen production not only in malignant tissues but also in non-malignant tissues. EST enzymatic activity has been reported previously in human breast carcinoma and normal breast tissues [18,19]. In addition, the concentration of E1S in breast cancer tissue has been reported to be significantly higher than plasma levels [20]. Results from our present study are in good agreement with previous findings and suggest that EST is involved in the inactivation of local estrogen not only in human breast cancer tissues but also in their surrounding non-malignant tissues.

In contrast to mRNA of STS and EST, mRNA of aromatase, 17β-HSD types I and II were detected only in small proportion of the patients, which are consistent with a previous report [14]. On the other hand, high protein expression and enzymatic activity of 17β-

HSD type I have been reported in breast cancer tissues [21]. Our results suggest a post-translational mechanism of protein stabilization of 17β-HSD.

In this study, we examined the breast tissues as a whole, which are composed of both stromal and parenchymal cells. It has been reported that STS is expressed exclusively in the parenchymal cells but aromatase is expressed in both stromal and parenchymal cells [13,22]. Therefore, it should be considered that mRNA levels of STS and aromatase measured in this study might be influenced by the ratio of stromal and parenchymal cells in the tumor tissues.

Furthermore, we measured the mRNA levels of the same nine genes in tumor tissues of 197 breast cancer patients, and analyzed relationship between the mRNA levels and the clinicopathological parameters.

Messenger RNA levels of STS and EST in tumor tissues elevated with increasing stage. These results may support the hypothesis that STS-mediated estrogen synthesis in the tumor tissues is becoming more important as the tumor progresses [23]. Messenger RNA levels of aromatase were not significantly affected by any clinicopathological factors except for clinical stage. Messenger RNA levels of aromatase in patients with clinical stage 3 and 4 were significantly higher than those in patients with clinical stage 2, when only detected samples ($n = 51$) were selected for the analysis. Thus, aromatase-mediated estrogen synthesis may also contribute to tumor progression in breast cancer, although additional analysis should be followed. We showed that mRNA levels of Cyclin D1 in tumor tissues were significantly higher in ER-positive patients. Cyclin D1 has been reported to be induced by estrogen and to activate ER-mediated transcription in a ligand-independent manner [24,25]. Our results may also support those findings.

Steroid sulfatase expression has been reported to be an independent predictor of recurrence [11]. Moreover overexpression of erbB2 has been reported to associate with poor prognosis of breast cancer patients [26]. Our results revealed that STS, ERβ and erbB2 mRNA expression levels in patients with recurrence were higher than those in patients without recurrence. These results may support previous findings; however, most patients analyzed in this study are still with recurrence-free (189 out of 197 patients) at present and alive within the follow-up period (37–2680 days, median: 750 days). Therefore, clinical importance of these factors in this study should be addressed after more prognostic data are accumulated.

In summary, we measured mRNA expression levels of 5 enzymes participating to estrogen synthesis *in situ* and 4 breast cancer-related proteins in 27 pairs of tumor and non-malignant tissues samples. Steroid sulfatase mRNA was more frequently detected in tumor tissue samples rather than in their non-malignant counterparts. Estrogen sulfotransferase was constantly expressed with high level not only in tumor tissues but also in their surrounding non-malignant counterparts. Messenger RNA of aromatase, 17β-HSD types I and II were detected only in small proportion of the patients. We also measured the mRNA expression levels of the same nine genes in tumor tissues of 197 breast cancer patients and analyzed relationship between the mRNA levels and the clinicopathological parameters. Messenger RNA levels of STS, aromatase, and erbB2 in tumor tissues increased as breast cancer progressed. Messenger RNA levels of STS, ERβ, and erbB2 in patients with recurrence were higher than those in patients without recurrence. Upregulation of STS expression plays an important role in tumor progression of human breast cancer and is considered to be responsible for estrogen production in tumor and surrounding tissues.

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