9. Sabnis G, Brodie A (2011) Adaptive changes results in activation of alternate signaling pathways and resistance to aromatase inhibitor resistance. Mol Cell Endocrinol 340:142–147
10. Martin LA, Farmer I, Johnston SR, Ali S, Dowsett M (2005) Elevated ERK1/ERK2/estrogen receptor cross-talk enhances estrogen-mediated signaling during long-term estrogen deprivation. Endocr Relat Cancer 12(Suppl 1):S75–S84
11. Yue W, Fan P, Wang J, Li Y, Santen RJ (2007) Mechanisms of acquired resistance to endocrine therapy in hormone-dependent breast cancer cells. J Steroid Biochem Mol Biol 106:02–110
12. Santen RJ, Song RX, Masamura S, Yue W, Fan P, Sogon T, Hayashi S, Nakachi K, Eguchi H (2008) Adaptation to estradiol deprivation causes up-regulation of growth factor pathways and hypersensitivity to estradiol in breast cancer cells. Adv Exp Med Biol 630:19–34
13. Hayashi S, Niwa T, Yamaguchi T (2009) Estrogen signaling pathway and its imaging in human breast cancer. Cancer Sci 100:1773–1778
14. Sikora MJ, Cordero KE, Larios JM, Johnson MD, Lippman ME, Rae JM (2009) The androgen metabolite 5alpha-androstane-3beta,17beta-diol (3betaAdiol) induces breast cancer growth via estrogen receptor: implications for aromatase inhibitor resistance. Breast Cancer Res Treat 115:289–296
15. Geisler J, Sasano H, Chen S, Purohit A (2011) Steroid sulfatase inhibitors: promising new tools for breast cancer therapy? J Steroid Biochem Mol Biol 125:39–45
16. Miller WR, Larionov AA (2012) Understanding the mechanisms of aromatase inhibitor resistance. Breast Cancer Res 14:201
17. Kuiper GG, Carlsson B, Grandien K, Enmark E, Häggblad J, Nilsson S, Gustafsson JA (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 138:863–870
18. Wang P, Wen Y, Han G, Sidhu KP, Zhu BT (2009) Characterization of the oestrogenic activity of non-aromatic steroids: are there male-specific endogenous oestrogen receptor modulators? Br J Pharmacol 158:1796–1807
19. Honma N, Saji S, Hirose M, Horiguchi S, Kuroi K, Hayashi S, Utsumi T, Harada N (2011) Sex steroid hormones in pairs of tumor and serum from breast cancer patients and pathobiological role of androstene-3β,17β-diol. Cancer Sci 102:1848–1854
20. Aspinall SR, Stamp S, Davison A, Shenton BK, Lennard TW (2004) The proliferative effects of 5-androstene-3β, 17β-diol and 5α-dihydrotestosterone on cell cycle analysis and cell proliferation in MCF7, T47D and MDAMB231 breast cancer cell lines. J Steroid Biochem Mol Biol 88:37–51
21. Purohit A, Woo LW, Potter BV (2011) Steroid sulfatase: a pivotal player in estrogen synthesis and metabolism. Mol Cell Endocrinol 340:154–160
22. Takagi K, Miki Y, Nagasaki S, Hirakawa H, Onodera Y, Akahira J, Ishida T, Watanabe M, Kimijima I, Hayashi S, Sasano H, Suzuki T (2010) Increased intratumoral androgens in human breast carcinoma following aromatase inhibitor exemestane treatment. Endocr Relat Cancer 17:415–430
23. Suzuki T, Darnel AD, Akahira JI, Ariga N, Ogawa S, Kaneko C, Takeyama J, Moriya T, Sasano H (2001) 5alpha-reductases in human breast carcinoma: possible modulator of in situ androgenic actions. J Clin Endocrinol Metab 86:2250–2257
24. Lorence MC, Murry BA, Trant JM, Mason JI (1990) Human 3β-hydroxysteroid dehydrogenase/delta 5 → 4isomerase from placenta: expression in nonsteroidogenic cells of a protein that catalyzes the dehydrogenation/isomerization of C21 and C19 steroids. Endocrinology 126:2493–2498
25. Pirog EC, Collins DC (1999) Metabolism of dihydrotestosterone in human liver: importance of 3α- and 3β-hydroxysteroid dehydrogenase. J Clin Endocrinol Metab 84:3217–3221
26. Hankinson SE, Willett WC, Manson JE, Colditz GA, Hunter DJ, Spiegelman D, Barbieri RL, Speizer FE (1998) Plasma sex steroid hormone levels and risk of breast cancer in postmenopausal women. J Natl Cancer Inst 90:1292–1299
27. Suzuki T, Miki Y, Nakamura Y, Ito K, Sasano H (2011) Steroid sulfatase and estrogen sulfotransferase in human carcinomas. Mol Cell Endocrinol 340:148–153
28. Hanamura T, Niwa T, Nishikawa S, Konno H, Gohno T, Tazawa C, Kobayashi Y, Kurosumi M, Takei H, Yamaguchi Y, Ito K, Hayashi S (2013) Androgen metabolite-dependent growth of hormone receptor-positive breast cancer as a possible aromatase inhibitor-resistance mechanism. Breast Cancer Res Treat 139:731–740
29. Matsumoto M, Yamaguchi Y, Seino Y, Hatakeyama A, Takei H, Niikura H, Ito K, Suzuki T, Sasano H, Yaegashi N, Hayashi S (2008) Estrogen signaling ability in human endometrial cancer through the cancer-stromal interaction. Endocr Relat Cancer 15:451–463
30. Gohno T, Seino Y, Hanamura T, Niwa T, Matsumoto M, Yaegashi N, Oba H, Kurosumi M, Takei H, Yamaguchi Y, Hayashi S (2013) Individual transcriptional activity of estrogen receptors in primary breast cancer and its clinical significance. Cancer Med. 1:328–337
31. Tokuda E, Seino Y, Arakawa A, Saito M, Kasumi F, Hayashi S, Yamaguchi Y (2012) Estrogen receptor-α directly regulates sensitivity to paclitaxel in neoadjuvant chemotherapy for breast cancer. Breast Cancer Res Treat 133:427–436
32. Kastner P, Krust A, Turcotte B, Stropp U, Tora L, Gronemeyer H, Chambon P (1990) Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. EMBO J 9:1603–1614
33. Hammond ME, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, Fitzgibbons PL, Francis G, Goldstein NS, Hayes M, Hicks DG, Lester S, Love R, Mangu PB, McShane L, Miller K, Osborne CK, Paik S, Perlmutter J, Rhodes A, Sasano H, Schwartz JN, Sweep FC, Taube S, Torlakovic EE, Valenstein P, Viale G, Visscher D, Wheeler T, Williams RB, Wittliff JL, Wolff AC (2010) American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. J Clin Oncol 28:2784–2795
34. Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, Dowsett M, Fitzgibbons PL, Hanna WM, Langer A, McShane LM, Paik S, Pegram MD, Perez EA, Press MF, Rhodes A, Sturgeon C, Taube SE, Tubbs R, Vance GH, van de Vijver M, Wheeler TM, Hayes DF (2007) American Society of Clinical Oncology/College of American Pathologists. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. Arch Pathol Lab Med 131:18–43
35. Specht K, Richter T, Müller U, Walch A, Werner M, Höfler H (2001) Quantitative gene expression analysis in microdissected archival formalin-fixed and paraffin-embedded tumor tissue. Am J Pathol 158(41):9–29
36. Cronin M, Pho M, Dutta D, Stephans JC, Shak S, Kiefer MC, Esteban JM, Baker JB (2004) Measurement of gene expression in archival paraffin-embedded tissues: development and performance of a 92-gene reverse transcriptase-polymerase chain reaction assay. Am J Pathol 164:35–42
37. Geisler J (2003) Breast cancer tissue estrogens and their manipulation with aromatase inhibitors and inactivators. J Steroid Biochem Mol Biol 86:245–253
38. Ortmann J, Prifti S, Bohlmann MK, Rehberger-Schneider S, Strowitzki T, Rabe T (2002) Testosterone and 5α-dihydrotestosterone inhibit in vitro growth of human breast cancer cell lines. Gynecol Endocrinol 16:113–120

39. Andò S, De Amicis F, Rago V, Carpino A, Maggiolini M, Panno ML, Lanzino M (2002) Breast cancer From estrogen to androgen receptor. Mol Cell Endocrinol 193:121–128
40. Labrie F, Luu-The V, Labrie C, Bélanger A, Simard J, Lin SX, Pelletier G (2003) Endocrine and intracrine sources of androgens in women: inhibition of breast cancer and other roles of androgens and their precursor dehydroepiandrosterone. Endocr Rev 24:152–182
41. Thomas JL, Bucholtz KM, Sun J, Mack VL, Kacsoh B (2009) Structural basis for the selective inhibition of human 3β-hydroxysteroid dehydrogenase 1 in human breast tumor MCF-7 cells. Mol Cell Endocrinol 301:174–182
42. Williams CJ, Barley VL, Blackledge GR, Rowland CG, Tyrrell CJ (1993) Multicentre cross over study of aminoglutethimide and trilostane in advanced postmenopausal breast cancer. Br J Cancer 68:1210–1215
43. James MR, Skaar TC, Lee RY, MacPherson A, Zwiebel JA, Ahluwalia BS, Ampy F, Clarke R (2001) Constitutive expression of the steroid sulfatase gene supports the growth of MCF-7 human breast cancer cells in vitro and in vivo. Endocrinology 142:1497–1505
44. Ishida H, Nakata T, Suzuki M, Shiotsu Y, Tanaka H, Sato N, Terasaki Y, Takebayashi M, Anazawa H, Murakata C, Li PK, Kuwabara T, Akinaga S (2007) A novel steroidal selective steroid sulfatase inhibitor KW-2581 inhibits sulfated-estrogen dependent growth of breast cancer cells in vitro and in animal models. Breast Cancer Res Treat 106:215–227
45. Hankinson SE, Willett WC, Manson JE, Colditz GA, Hunter DJ, Spiegelman D, Barbieri RL, Speizer FE (1998) Plasma sex steroid hormone levels and risk of breast cancer in postmenopausal women. J Natl Cancer Inst 90:1292–1299
46. Ruder HJ, Loriaux L, Lipsett MB (1972) Estrone sulfate: production rate and metabolism in man. J Clin Invest 51:1020–1033
47. Stanway SJ, Purohit A, Woo LW, Sufi S, Vigushin D, Ward R, Wilson RH, Stanczyk FZ, Dobbs N, Kulinskaya E, Elliott M, Potter BV, Reed MJ, Coombes RC (2006) Phase I study of STX 64 (667 Coumate) in breast cancer patients: the first study of a steroid sulfatase inhibitor. Clin Cancer Res 12:1585–1592
48. Purohit A, Foster PA (2012) Steroid sulfatase inhibitors for estrogen- and androgen-dependent cancers. J Endocrinol 212:99–110
49. Chanplakorn N, Chanplakorn P, Suzuki T, Ono K, Chan MS, Miki Y, Saji S, Ueno T, Toi M, Sasano H (2010) Increased estrogen sulfatase (STS) and 17β-hydroxysteroid dehydrogenase type 1(17β-HSD1) following neoadjuvant aromatase inhibitor therapy in breast cancer patients. Breast Cancer Res Treat 120:639–648

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ORIGINAL ARTICLE

Detection of Estrogen-Independent Growth-Stimulating Activity in Breast Cancer Tissues: Implication for Tumor Aggressiveness

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Abstract Estrogen and various growth factors affecting tumor behavior are present in the breast cancer microenvironment, but their comprehensive effects and signal crosstalks are different in each case. However, there is no system to evaluate the factors, detected in individual breast cancer cases, that regulate ER activity and tumor progression. In this study, we analyzed the effects of individual breast cancer extracts by our original system using an estrogen-signal reporter cell line, MCF-7-E10, which we previously established. MCF-7-E10 cell line is stably transfected by an estrogen response element (ERE)-green fluorescent protein (GFP) gene; it expresses GFP when estrogen receptors (ERs) are activated by estrogen or growth factor signal-mediated ER phosphorylation. Using this cell line, we analyzed the comprehensive effects of factors derived from breast cancer tissues on ER activity and growth of MCF-7-E10 cells for each case. We also analyzed relationships between these activities and clinicopathologic characteristics of patients who provided cancer specimens. The breast cancer extracts, which reflect the combined activities of growth factors present in individual cases, stimulated MCF-7-E10 cell growth in an estrogen-independent manner, and specifically stimulated growth of other breast cancer cell lines, regardless of ER expression. High growth-promoting activities were seen in tumor regions of specimens with tumors > 10 mm in size, HER2 intrinsic subtype, and scirrhous and solid-tubular carcinoma histological subtypes. Anti-human hepatocyte growth factor (HGF) antibody and an inhibitor for insulin-like growth factor-1 (IGF-1) receptor inhibited MCF-7-E10 cell growth by the breast cancer extracts, indicating that signal pathways via HGF or IGF-1 receptor significantly affect breast cancer. These data suggest that growth factors other than estrogen in the tumor extract significantly affect breast cancer aggressiveness in an estrogen-independent manner, and could be useful therapeutic targets.

Keywords Tumor microenvironment · Estrogen-independent · HGF · IGF1-R · Breast cancer

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Background

The tumor microenvironment is enriched in factors such as growth factors, cytokines and chemokines, and critically affects initiation and progression of various tumor types [1–5]. For postmenopausal women with low levels of plasma estrogen, breast cancer growth and progression are mainly caused by estrogen produced locally in the tumor microenvironment [6–8]. Intratumoral production of estrogen is induced by aromatase, a key enzyme in estrogen biosynthesis, which is expressed by carcinoma-associated stromal fibroblasts [7–10]. Aromatase is a target of endocrine therapy for breast

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cancers; aromatase inhibitors attenuate estrogen biosynthesis in treating hormone-responsive breast cancer [11, 12]. Estrogen stimulates breast cancer growth via expression of a diverse set of growth-related genes in tumor cells, and through activation of estrogen receptor (ER), a transcription factor [13, 14]. ERα is a primary predictive marker for hormonal therapy in breast cancer, but approximately one-third of ER + patients do not respond to this therapy, suggesting that ERα is not a perfect predictor for hormonal therapy. To shed light on these issues, and to study the molecular basis for breast cancer, we first focused on analysis of estrogen signals by development of a custom-made cDNA microarray, and provided novel diagnostic and prognostic estrogen-induced genes [15–17].

In addition to the genomic pathway, estrogen induces non-genomic pathways by interacting with signal cascades for growth factors [4, 13], such as epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1), which activate ERα in an estrogen-independent manner by phosphorylating several ERα sites using their downstream signal kinases, including MAPK and PI3K [4, 5, 18–20]. Growth factors are produced by malignant cells themselves, adjacent tumor stromal fibroblasts and inflammatory cells in the microenvironment.

To analyze the carcinoma-associated fibroblasts-induced ER activation in individual breast cancers, we established an estrogen response element (ERE)-green fluorescent protein (GFP) assay system. It allows us to detect estrogen- and phosphorylation-dependent ER-activating ability of stromal fibroblasts adjacent to tumor cells under coculture with MCF-7-E10 cells, a clone of MCF-7 stably transfected with the *ERE-GFP* gene [20]. Using this system, we examined relationships between ER-activating ability of stromal fibroblasts and clinicopathological characteristics. We found that, although ER-activating abilities of stromal fibroblasts vary among breast cancers, they are higher in breast cancers from postmenopausal patients than in those from premenopausal patients [20]. This is in accordance with the fact that intratumoral estrogen production causes progression of postmenopausal breast cancers [6–8]. ER-activating abilities of fibroblasts in grade 3 breast cancers are lower than in grade 1 breast cancers, suggesting that the grade 3 microenvironment stimulates proliferation of breast cancer cells via an estrogen-independent pathway [20].

In the breast cancer microenvironment, various growth factors and cytokines reportedly interact to control tumor growth, as described above. However, their significance in tumor growth in vivo and in response to hormonal therapy are unclear. To investigate the role of breast cancer-derived factors on breast cancer growth, we studied effects of the supernatants of minced breast cancer tissues on MCF-7-E10 cell growth. Tissue supernatant, unlike tissue extract or conditioned medium of tissue prepared after culture for several days in vitro, reflects the comprehensive effects of factors detected in the tumor in vivo. In addition to estrogen-related signals, we found that the breast cancer-derived factors effectively stimulate MCF-7-E10 cell growth via an estrogen-independent pathway.

Methods

Cells & Cell Culture

Cell lines used in this study were cultured in RPMI1640 medium (GIBCO) supplemented with 10 % FCS (Tissue Culture Biologicals) at 37 °C in a humidified atmosphere of 5 % CO_2. We previously established the estrogen-signal reporter cell line MCF-7-E10 derived from MCF-7 cells by stable transfection with an *ERE-GFP* reporter plasmid [20]. To analyze the effect of breast cancer tissue supernatant (BCTS) on ER activity in MCF-7-E10 cells, cells were precultured in estrogen-deprived medium (phenol red-free RPMI1640 medium supplemented with 10 % dextran-coated, charcoal-treated FCS) for 3 days.

Preparation of BCTS

Breast cancer specimens were processed within 1 h after surgical resection. After being weighed, specimens were transferred to tubes containing phenol red- and serum-free RPMI 1640 medium at 100 mg/ml, and minced to particles < ~1 mm^3 in size. The suspension was centrifuged (600 × g, 10 min, 4 °C) and the supernatant was further centrifuged (12, 000 × g, 10 min, 4 °C) to obtain BCTS. The protein concentration of each sample was determined using BCA Protein Assay Reagent (PIERCE).

Human breast cancer tissues were obtained by surgery at the Saitama Cancer Center Hospital (Saitama, Japan) after informed consent had been obtained from the patients. The Saitama Cancer Center Ethics Committee approved this study. In the clinicopathological classifications of the patients (Table 1), ER and progesterone receptor (PgR) status was determined using monoclonal anti-ERα antibody 1D5 (Dako, Glostrup, Denmark) and monoclonal anti-PgR antibody PgR636 (Dako), and evaluated on the basis of Allred scoring [21]. HER2 protein expression was scored as 0, 1+, 2+ or 3+ using the HercepTest™ (Dako); *HER2* genome status was evaluated by fluorescent in situ hybridization (FISH) using PathVysion HER-2 DNA Probe Kit (Abbott Laboratories, Abbott Park, IL, USA). According to the ASCO/CAP guidelines [22], absolute *HER2* gene/chromosome 17 copy number ratios

Table 1 Clinical characteristics of patients

	No. of patients[a]
Age (y)	
< 51	26
≥ 50	65
Unknown	2
Menopausal status	
Pre	28
Post	64
Male	1
Tumor diameter (cm)	
≤ 1	15
> 1	75
Unknown	3
ER	
Positive	69
Negative	24
PgR	
Positive	52
Negative	41
HER2	
0	37
1	31
2	10
3	13
Unknown	2
Stage	
0	1
I	37
II	42
III	11
Unknown	2
Histology	
Scirrhous	41
Solidtubular	23
Papillotubular	13
Mucinous	7
Apocrine	2
DCIS	3
Unknown	4
Grade	
1	18
2	17
3	47
Unknown	11
Nodal status	
Negative	38
Positive	53
Unknown	2

[a] Total number of patients was 93

greater than 2.2 and less than 1.8 indicated *HER2* amplification (positive) and *HER2* non-amplification (negative), respectively. Histologic grading was evaluated according to the Elston and Ellis grading scheme [23].

Cell Growth Assay

After 3 days of culture in estrogen-deprived medium, cells were seeded at 1×10^3/150 μl in a 96-well multi-dish culture plate, or at 1×10^4/1 ml in a 24-well plate, with or without BCTS at indicated protein concentrations for 4 days. Viable cells were examined using a Cell Counting Kit-8 assay according to manufacturer's instructions (Dojindo Laboratories, Japan).

Evaluation of ER Activity

ER activities in MCF-7-E10 cells, which had been transfected with *ERE-GFP*, after incubation with BCTS or E2, were monitored through GFP expression [20]. To quantify GFP expression, cells expressing GFP were counted under a fluorescence microscope after the cells were harvested by treatment with trypsin. Data are presented as percentage of cells expressing GFP.

Quantification of Growth Factors in BCTS by ELISA

Human EGF and IGF-1 levels in BCTS were quantified by ELISA using Quantikine (R&D Systems, MN, USA) specific for each growth factor.

Materials

Unless otherwise stated, all other materials were from Sigma-Aldrich Inc. (St. Louis, MO, USA). Inhibitors for EGF receptor and IGF receptor, and normal mouse IgG were from Calbiochem. Mouse anti-human HGF monoclonal antibody was from the Institute of Immunology (Tokyo, Japan). Mouse IgG1 antibody (Chemicon International, CA, USA) was used as an isotype control. IGF-1 receptor inhibitor, AG1024, and EGF receptor inhibitor, AG1478, were from Chemicon International.

Statistical Analysis

Statistical analyses were performed using the Stat Flex version 6.0 software program (Artech Co., Ltd., Osaka, Japan). In comparisons among groups, ANOVA and two-sample *t*-tests were used to assess the statistical significance of differences.

Data are expressed as means ± S.D. $P<0.05$ was considered statistically significant.

Results

BCTS Stimulates Breast Cancer Cell Growth in Both Estrogen-Dependent and -Independent Manners

In the tumor microenvironment, many growth factors, cytokines and chemokines directly and indirectly control growth. To study their comprehensive influence on breast cancer aggressiveness, we first analyzed effects of BCTS on MCF-7-E10 cell growth (Fig. 1), which allowed us to examine the total effect of breast cancer-derived factors secreted from tumor and stromal cells, as they exist in vivo, on growth and estrogen-related signals of breast cancer cells. BCTS dose-dependently stimulated MCF-7-E10 cell growth (Fig. 1a). Although activities varied among specimens, more than 60 % showed higher growth-stimulating activity than with estrogen (Fig. 1b).

To examine the specificity of target cells, we studied the effect of BCTS on growth of other tumor cell lines, including a breast cancer cell line, T47D, a lung adenocarcinoma cell line, PC9, and a cervical cancer cell line, HeLa (Fig. 2a). The growth of T47D, another ER + human breast cancer cell line, was stimulated by BCTS while growth of PC9 was not increased. HeLa cell growth was rather inhibited by BCTS. The growth of MDA-MB-231 cells, an ER– human breast cancer cell line, was also stimulated by the extracts (data not shown). These results suggest that BCTS specifically stimulated breast cancer cell growth regardless of ER expression.

Next, to see whether growth-stimulating activity in the tissue supernatant affected only the tumoral region, we analyzed extracts of tumoral regions and non-tumoral regions 2 cm distal to the tumor. The tumoral regions had more growth-stimulating activity than the non-tumoral regions (Fig. 2b), suggesting that the tumoral regions have an abundance of growth-stimulating activities for breast cancer cells.

To see if ER activation was required for BCTS-induced growth stimulation, we analyzed GFP expression in MCF-7-E10 cells, and found growth stimulation was not necessarily accompanied by ER activation (Fig. 3a). We next examined effects of anti-estrogen agents such as tamoxifen and fulvestrant on BCTS-induced growth stimulation, and found that high growth-stimulating activities were resistant to fulvestrant (Fig. 3b) and tamoxifen (Fig. 3c). These results indicate that, in addition to an ER-dependent pathway, BCTS stimulates breast cancer growth via an ER-independent pathway.

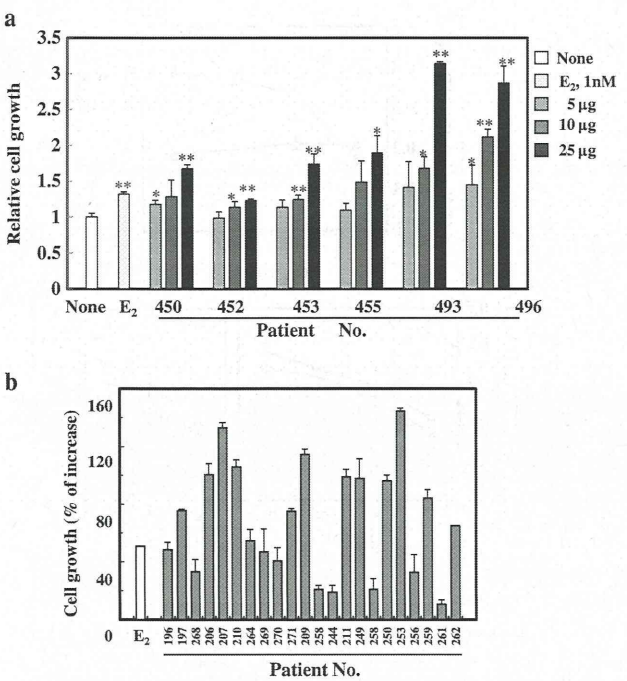

Fig. 1 BCTS effectively stimulated growth of MCF-7-E10 cells. After 3 days of culture in estrogen-deprived medium, MCF-7-E10 cells were cultured with breast cancer tissue supernatant at the indicated protein concentrations (**a**) or at 25 μg (**b**) in total 150 μl medium per well in 96-well plate for 4 days. The viable cells were examined using a Cell Counting Kit-8 assay. Values relative to control are shown. Data are presented as mean ± SD of triplicate determinations. *, $P<0.05$; **, $P<0.01$

Growth-Stimulating Activity Correlated with Clinicopathological Characteristics

We analyzed the relationships between ER-independent growth-stimulating activity detected in BCTS and clinicopathologic characteristics of the specimens' donors (Fig. 4). Although BCTS growth-stimulating activity did not correlate with expression of ERα or PgR, stage, menopausal status, grade or nodal status (data not shown), specimens from tumors larger than 10 mm showed higher growth-stimulating activity than those smaller than 10 mm (Fig. 4a). Breast cancers are categorized into four intrinsic subtypes according to gene-expression profile: luminal A (ER + and/or PgR+, HER2–), luminal B (ER + and/or PgR+, HER2+), HER2 (ER–, PgR–, HER2+) and basal-type (ER–, PgR–, HER2–) [24, 25]. BCST derived from HER2 subtype showed slightly or significantly higher growth-stimulating activity than that from luminal B or basal types, respectively (Fig. 4b), suggesting that the tumor extracts of HER2 subtype have an abundance of growth factors stimulating their own receptors, including those of the ERBB family.

We next analyzed relationships between HER2 expression and growth-stimulating activity in ER– breast cancers, and found that the cases with high growth-stimulating activity differed significantly from those with low activity in terms

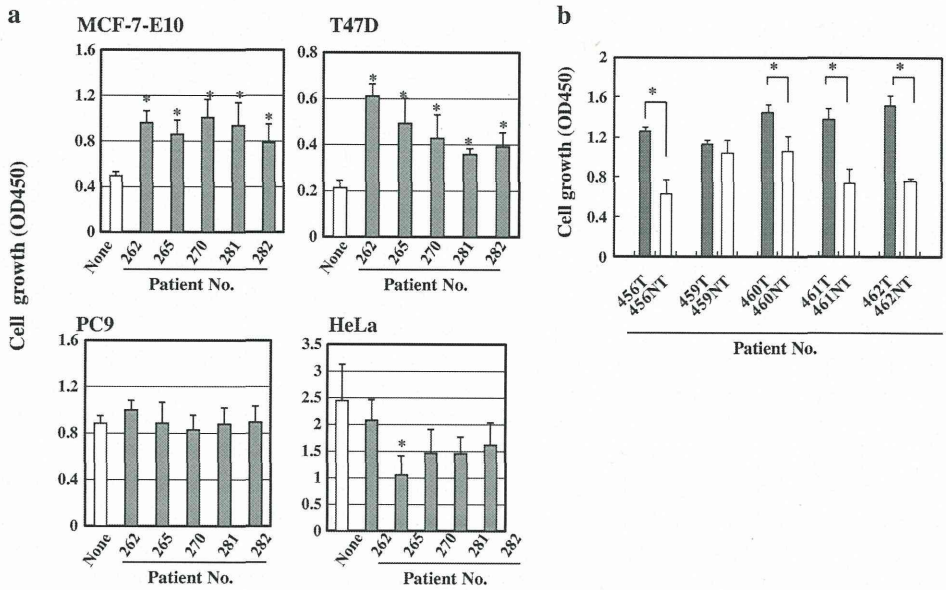

Fig. 2 Specificity of cell growth-stimulating activity detected in BCTS. **a** BCTS at 25 μg protein concentration in 150 μl medium per well in 96-well plate effectively stimulated growth of breast cancer cell lines, MCF-7-E10 and T47D which were precultured in estrogen-deprived medium for 3 days. The viable cells were examined using a Cell Counting Kit-8 assay. **b** Specificity for BCTS-derived region. T, tissue supernatant derived from tumor region; NT, tissue supernatant derived from the region 2 cm distal to the tumor region. Data are presented as mean ± SD of triplicate experiments. *, $P<0.05$

of HER2 expression; more specimens with high HER2 scores (score 3) were seen among cases with high activity (Fig. 4c). This difference could not be observed for ER + breast cancers.

Breast cancers have histological types that reflect biological characteristics. Invasive ductal carcinoma can be classified into three subtypes—papillotubular, solid-tubular and

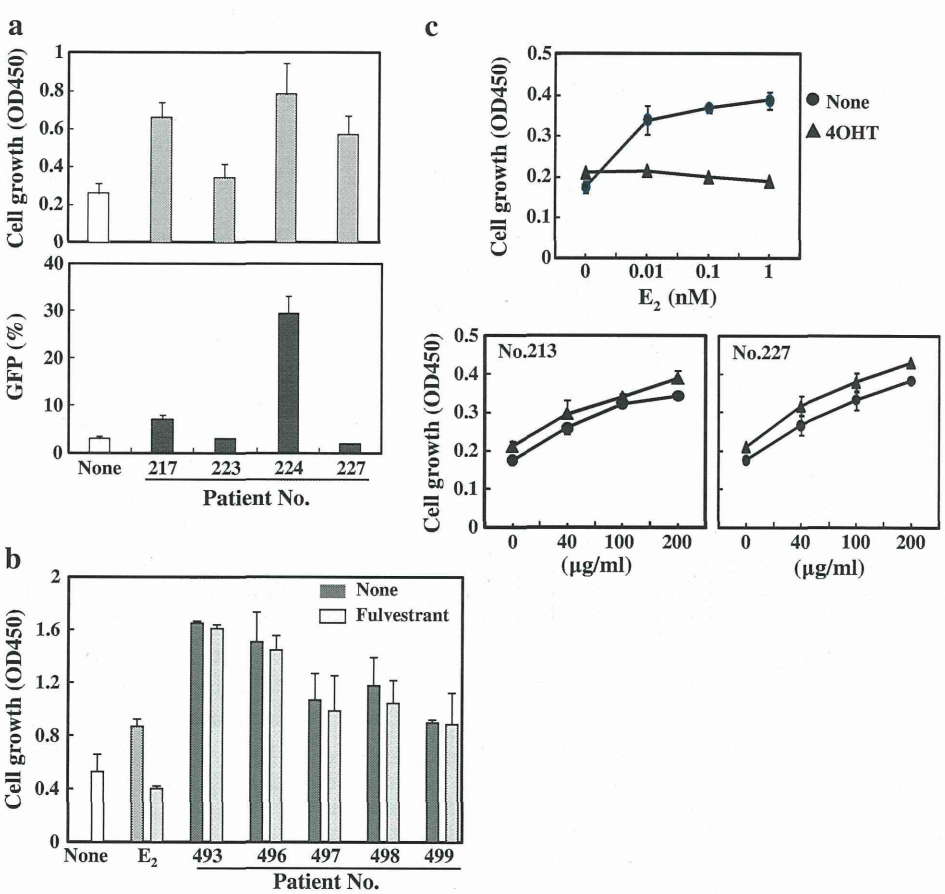

Fig. 3 BCTS stimulated growth of MCF7-E10 cells in an estrogen-independent manner. **a** MCF7-E10 cells were cultured with BCTS at 25 μg protein concentration in 150 μl medium per well in 96-well plate for 4 days. Cell growth was examined using a Cell Counting Kit-8 assay and ER activities are shown as the percentage of MCF-7-E10 cells expressing GFP. **b**, **c** MCF-7-E10 cells were cultured with BCTS at 25 μg protein concentration in 150 μl medium per well in 96-well plate or the indicated concentrations in the presence or absence of anti-estrogen agents, fulvestrant (1 μM) or 4-hydroxy tamoxifen (4OHT, 1 μM), for 4 days. 17β-Estradiol (E_2) was also tested at 1 nM or the indicated concentrations. Cell growth was examined using a Cell Counting Kit-8 assay. Data are presented as mean ± SD of triplicate experiments

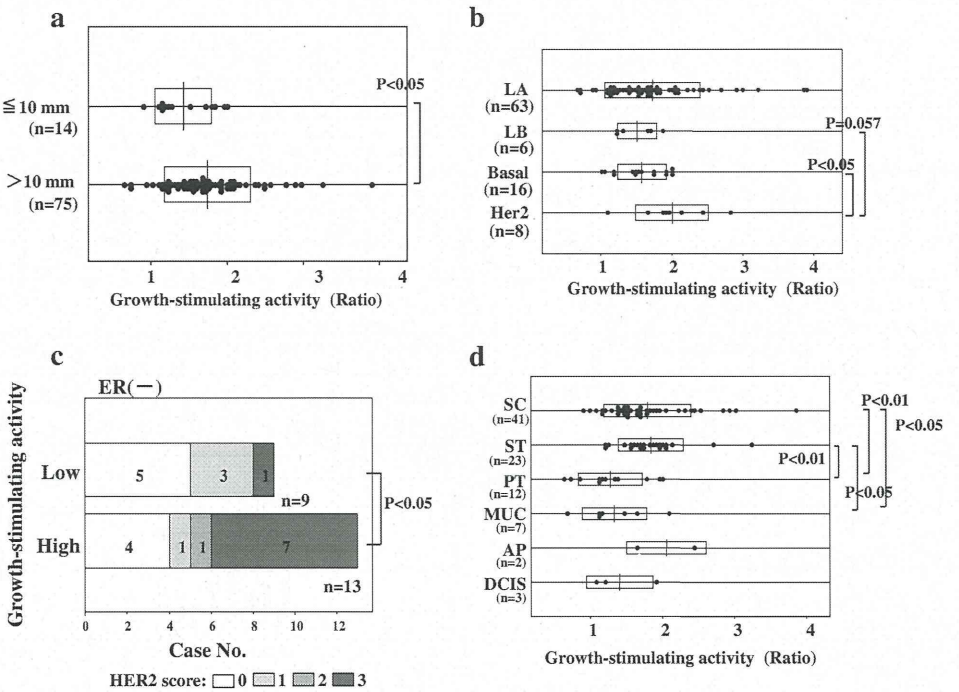

Fig. 4 Correlations between growth-stimulating activity and clinicopathological characteristics, intrinsic subtypes and histological subtypes. MCF7-E10 cells were cultured with BCTS at 25 μg protein concentration in 150 μl medium per well in 96-well plate for 4 days. Cell growth was examined as described in Materials and Methods for triplicate experiments, and the growth-stimulating activities are shown as the ratios calculated relative to the control. Data are presented as mean ± SD of triplicate experiments. High growth-stimulating activity in specimens was associated with tumor size (**a**), intrinsic subtype (**b**), HER2 expression in ER-negative breast cancer (**c**) or histological classifications (**d**). Differences between groups were determined by two-sample *t*-test. $P<0.05$ was considered statistically significant

scirrhous carcinoma—which are related to prognosis. We previously reported their relative overall survival rates as papillotubular carcinoma > solid-tubular carcinoma > scirrhous carcinoma [26]. The more aggressive scirrhous carcinoma and solid-tubular carcinoma show higher growth-stimulating activity than do papillotubular carcinoma and mucinous types (Fig. 4d), suggesting that growth-stimulating activity is related to aggressiveness in breast cancer.

Growth Factors in BCTS Promote MCF-7-E10 Cell Growth

Growth-stimulating activity was heat labile and detectable in the fraction with an MW greater than 5 kDa (data not shown), suggesting that it could be derived from proteinous factors. Among various factors in the tumor microenvironment, HGF derived from stromal fibroblasts has been reported to stimulate growth of mouse mammary tumor cells in primary culture [27]; EGF and IGF-1 are known to activate ER via phosphorylation [18, 19]. To analyze the participation of these growth factors in tumor growth-stimulating activities found in BCTS, we first examined the effect of anti-HGF antibody on them. As shown in Fig. 5a, anti-HGF antibody, but not control IgG, effectively inhibited extract-stimulated growth of MCF-7-E10 cells. MCF-7 cells reportedly express c-Met, a receptor for HGF.

We next analyzed the roles of EGF and IGF-1, using the inhibitors specific for their receptors. IGF-R inhibitor dose-dependently inhibited the growth of MCF-7-E10 cells while EGF-R inhibitor, in contrast, stimulated their growth (Fig. 5b, c).

Finally, we analyzed growth factors present in BCTS using the enzyme immunoassay. HGF was detected in more than 70 % of the tested samples, whereas EGF was detected only in 3 out of 25 samples (Fig. 5d). Although the analysis using IGF-1R inhibitor suggested involvement of IGF-1 in the growth-stimulating effect of BCTS as described above, IGF-1 could not be detected in the enzyme immunoassay. This might be because of the immunoassay's sensitivity, or because other ligands for IGF-1R (such as IGF-II, insulin or unknown factors) might have been present in the tumor extracts. These results suggest that signal pathways via HGF or IGF-1R play a significant role in promoting the growth of breast cancer cells.

Discussion

The tumor microenvironment is apparently associated with important aspects of epithelial solid tumor progression, including tumor growth, angiogenesis and metastasis. In the tumor microenvironment, growth factors such as EGF, IGF-1, transforming growth factor-α, transforming growth factor β

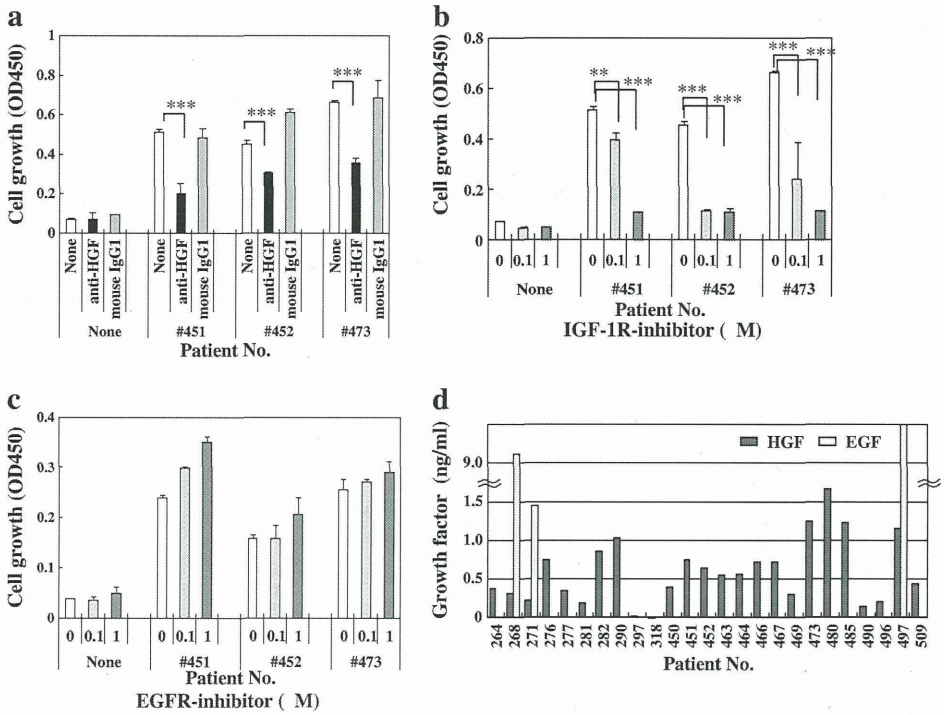

Fig. 5 Detection of growth factors involved in growth-promoting activity for MCF-7-E10 cells in BCTS. MCF-7-E10 cells were incubated with BCTS in the presence of anti-HGF antibody (**a**), AG1024, IGF-1R inhibitor (**b**), or AG1478, EGFR inhibitor (**c**), at the indicated concentrations. For anti-HGF antibody treatment, BCTS was pre-incubated with anti-HGF antibody for 30 min at room temperature and was then used for assay. Mouse IgG1 antibody was used as an isotype control. **d** The concentrations of HGF and EGF detected in BCTS were analyzed by immunoassay using Quantikine (R&D Systems, MN, USA). **, $P<0.01$, ***, $P<0.001$

and stromal-derived factor-I reportedly affect breast cancer growth, directly or indirectly [1, 3, 4]; however, the combined effects of these factors and their signal interactions in vivo are unclear. In this study, using the supernatant of breast cancer tissues, we analyzed the comprehensive effects of breast cancer-derived factors and found that BCTS effectively and specifically stimulated breast cancer cell growth. In addition to estrogen, which is locally produced in the microenvironment in breast cancers of postmenopausal patients [6, 8], our results suggest that the tumor extracts also stimulated breast cancer cell growth in an estrogen-independent manner, as anti-estrogen agents such as tamoxifen and fulvestrant did not inhibit the effect of BCTS. Furthermore, clinicopathological data and BCTS-associated growth-stimulation correlated with tumor size and HER2 expression, indicating the physiological significance of growth-stimulating activity in BCTS. Thus, BCTS offers an appropriate means to analyze the combined effect of the breast cancer-derived factors on tumor cell behavior.

Although many growth factors might be present in BCTS, we found HGF and IGF-1R-related signals to affect the growth-stimulating activity of BCTS, because it was suppressed by anti-HGF antibody and IGF-1R inhibitor. HGF was detected in tissue extracts of more than 70 % of breast cancer specimens whereas EGF was detected in only 12 % (Fig. 5d). The growth-stimulating activities did not always correlate with HGF concentrations (data not shown), but this is expected, as growth-stimulating activities in the supernatant are derived from the signal cross-talks of several factors. HGF, which acts through its receptor MET, is a multifunctional cytokine that induces cell survival, growth, differentiation and motility in most solid human cancers including colorectal, renal and breast cancers [28]. In normal epithelial cells, HGF, in combination with other growth factors, promotes mammary ductal morphogenesis [29]. Overexpression of both HGF and MET have been frequently reported in breast cancers, and are associated with poor prognosis [30]. HGF reportedly stimulates breast cancer growth in a paracrine fashion, in that HGF is produced primarily by stromal fibroblasts and acts on epithelial cells through its receptor MET [27, 31]. Stromal fibroblasts from breast cancer tissue produce large amounts of HGF compared with normal fibroblasts [30]. A c-Met-targeted therapy, ARQ197—which selectively targets c-Met tyrosine kinase—is currently in a phase II clinical trial [32]; SGX523—a novel ATP-competitive inhibitor, that is exquisitely selective for inhibition of MET-mediated signaling—is also being developed [33].

We found that IGF-1R signaling mediated the growth-stimulating activity of BCTS, because IGF-1R-specific inhibitor decreased the growth-stimulating effect of BCTS. IGF-1R-related signals are widely shown to induce cell proliferation and survival in breast cancer [34–36]; IGF-R1 activation protects breast cancer cells from apoptosis induced by various anticancer drugs [37]. While BCTS stimulated growth of MCF-7-E10 cells in an estrogen-independent manner, functional interactions between estrogen and IGF-1R signaling

pathways, including Ras/MAPK and PI3K/Akt have been reported [38] Estrogen also up-regulates IGF-1R expression in breast cancer [36]. However, we could not detect IGF-1 and stromal cell-derived growth factor-1alpha in BCTS (data not shown), possibly because of the limit of sensitivity by the immunoassay used in our study; or that other ligands may be present in the breast cancer microenvironment that activate IGF-1R—including IGF-II, insulin and unknown factors [39]. Indeed, overexpression of IGF-1R in MCF-7 cells has been shown to induce IGF-1R tyrosine kinase activation in the absence of exogenous IGF-1 [40].

These results suggest that signaling pathways via HGF/c-Met or IGF-1R significantly affect breast cancer cell growth. However, growth-stimulating activity found in BCTS might be derived from orchestrated signal crosstalks of several factors, because recombinant growth factors, including HGF and IGF-1, could not induce MCF-7-E10 cell growth when used alone. Further investigations of these activities and the identification of the cellular sources of the growth factors are needed to identify the mechanisms of the growth-stimulating effect of breast cancer tissue supernatant, which may help design more effective targeted therapies for breast cancer.

Conclusions

The breast cancer microenvironment provides estrogen and growth factors that affect tumor behavior, but the comprehensive effects of these factors, including signal crosstalk, on progression of breast cancer remain unclear. Using an estrogen-signal reporter cell line, MCF-7-E10, stably transfected with the ERE-GFP gene, we analyzed the effect of factors present in breast cancer tissues to reflect the in vivo status of individual cases. We found that they stimulated growth of MCF-7-E10 cells in an estrogen-independent manner, and that growth-promoting activity is related to aggressiveness in breast cancer. Moreover, signal pathways via HGF and IGF-1 receptor were involved in these activities. Our study strongly suggests that the evaluation of comprehensive tumor-promoting activity for individual breast cancers is important in determining appropriate therapy.

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Authors' contributions YY and SH were involved in experimental design, performed all experiments, and drafted the manuscript. YS assisted in experiments and performed statistical analysis of the data. HT participated in acquisition and interpretation of the clinical data of patients. MK participated in experimental design and histological evaluation. All authors contributed to the analysis of data and approved the final manuscript.

References

1. Allinen M, Beroukhim R, Cai L, Brennan C, Lahti-Domenici J, Huang H, Porter D, Hu M, Chin L, Richardson A et al (2004) Molecular characterization of the tumor microenvironment in breast cancer. Cancer Cell 6:17–32
2. Mueller MM, Fusenig NE (2004) Friends or foes: bipolar effects of the tumour stroma in cancer. Nat Rev Cancer 4:839–849
3. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA (2005) Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell 121:335–348
4. Yamaguchi Y (2007) Microenvironmental regulation of estrogen signals in breast cancer. Breast Cancer 14:175–181
5. Yamaguchi Y, Hayashi S (2009) Estrogen-related cancer microenvironment of breast carcinoma. Endocr J 56:1–7
6. O'Neill JS, Miller WR (1987) Aromatase activity in breast adipose tissue from women with benign and malignant breast diseases. Br J Cancer 56:601–604
7. Santen RJ, Santner SJ, Pauley RJ, Tait L, Kaseta J, Demers LM, Hamilton C, Yue W, Wang JP (1997) Estrogen production via the aromatase enzyme in breast carcinoma: which cell type is responsible? J Steroid Biochem Mol Biol 61:267–271
8. Simpson ER, Davis SR (2001) Minireview: aromatase and the regulation of estrogen biosynthesis–some new perspectives. Endocrinology 142:4589–4594
9. Zhao Y, Agarwal VR, Mendelson CR, Simpson ER (1997) Transcriptional regulation of CYP19 gene (aromatase) expression in adipose stromal cells in primary culture. J Steroid Biochem Mol Biol 61:203–210
10. Smith IE, Dowsett M (2003) Aromatase inhibitors in breast cancer. N Eng J Med 348:2431–2442
11. Dowsett M, Cuzick J, Ingle J, Coates A, Forbes J, Bliss J, Buyse M, Baum M, Buzdar A, Colleoni M et al (2010) Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. J Clin Oncol 28:509–518
12. Hayashi S, Yamaguchi Y (2005) Estrogen signaling and prediction of endocrine therapy. Cancer Chemother Pharmacol 56:27–31
13. Hayashi S, Niwa T, Yamaguchi Y (2009) Estrogen signaling pathway and its imaging in human breast cancer. Cancer Sci 100:1773–1778
14. Hayashi S (2004) Prediction of hormone sensitivity by DNA microarray. Biomed Pharmacother 58:1–9
15. Inoue A, Yoshida N, Omoto Y, Oguchi S, Yamori T, Kiyama R, Hayashi S (2002) Development of cDNA microarray for expression profiling of estrogen-responsive genes. J Mol Endocrinol 29:175–192
16. Inoue A, Omoto Y, Yamaguchi Y, Kiyama R, Hayashi S (2004) Transcription factor EGR3 is involved in the estrogen-signaling pathway in breast cancer cells. J Mol Endocrinol 32:649–666
17. Yoshida N, Omoto Y, Inoue A, Eguchi H, Kobayashi Y, Kurosumi M, Saji S, Suemasu K, Okazaki T, Nakachi K et al (2004) Prediction of prognosis of estrogen receptor-