HORM CANC (2013) 4:1–11
DOI 10.1007/s12672-012-0126-6

ORIGINAL PAPER

Intratumoral Estrogen Concentration and Expression of Estrogen-Induced Genes in Male Breast Carcinoma: Comparison with Female Breast Carcinoma

Kiyoshi Takagi · Takuya Moriya · Masafumi Kurosumi · Kimako Oka · Yasuhiro Miki · Akiko Ebata · Takashi Toshima · Shoji Tsunekawa · Hiroyuki Takei · Hisashi Hirakawa · Takanori Ishida · Shin-ichi Hayashi · Junichi Kurebayashi · Hironobu Sasano · Takashi Suzuki

Received: 10 August 2012 / Accepted: 8 October 2012 / Published online: 18 October 2012

Abstract It is speculated that estrogens play important roles in the male breast carcinoma (MBC) as well as the female breast carcinoma (FBC). However, estrogen concentrations or molecular features of estrogen actions have not been reported in MBC, and biological significance of estrogens remains largely unclear in MBC. Therefore, we examined intratumoral estrogen concentrations, estrogen receptor (ER) α/ERβ status, and expression profiles of estrogen-induced genes in MBC tissues, and compared these with FBC. 17β-Estradiol concentration in MBC (n=4) was significantly (14-fold) higher than that in non-neoplastic male breast (n=3) and tended to be higher than that in FBC (n=7). Results of microarray analysis clearly demonstrated that expression profiles of the two gene lists, which were previously reported as estrogen-induced genes in MCF-7 breast carcinoma cell line, were markedly different between MBC and FBC. In the immunohistochemistry, MBC tissues were frequently positive for aromatase (63 %) and 17β-hydroxysteroid dehydrogenase type 1 (67 %), but not for steroid sulfatase (6.7 %). A great majority (77 %) of MBC showed positive for both ERα and ERβ, and its frequency was significantly higher than FBC cases. These results suggest that estradiol is locally produced in MBC

K. Takagi (✉) · T. Suzuki
Department of Pathology and Histotechnology,
Tohoku University Graduate School of Medicine,
2-1 Seiryo-machi, Aoba-ku,
Sendai, Miyagi-ken 980-8575, Japan
e-mail: k-takagi@med.tohoku.ac.jp

T. Moriya
Department of Pathology 2, Kawasaki Medical School,
Kurashiki, Japan

M. Kurosumi
Department of Pathology, Saitama Cancer Center,
Saitama, Japan

K. Oka · H. Hirakawa
Department of Surgery, Tohoku Kosai Hospital,
Sendai, Japan

Y. Miki · H. Sasano
Department of Anatomic Pathology,
Tohoku University Graduate School of Medicine,
Sendai, Japan

A. Ebata · T. Ishida
Department of Surgical Oncology,
Tohoku University Graduate School of Medicine,
Sendai, Japan

T. Toshima
Department of Surgery, Tohoku Rosai Hospital,
Sendai, Japan

S. Tsunekawa
Department of Surgery, Kansai Electric Power Hospital,
Osaka, Japan

H. Takei
Division of Breast Surgery, Saitama Cancer Center,
Saitama, Japan

S.-i. Hayashi
Department of Molecular and Functional Dynamics,
Tohoku University Graduate School of Medicine,
Sendai, Japan

J. Kurebayashi
Department of Breast and Thyroid Surgery,
Kawasaki Medical School,
Kurashiki, Japan

H. Sasano
Department of Pathology, Tohoku University Hospital,
Sendai, Japan

tissue by aromatase. Different expression profiles of the estrogen-induced genes may associate with different estrogen functions in MBC from FBC, which may be partly due to their ERα/ERβ status.

Introduction

Male breast carcinoma (MBC) is an uncommon disease, and its incidence is less than 1 % of that in female breast carcinoma (FBC). However, it has been increasing in recent years [1]. Because of the low incidence, MBC has not been studied well, and limited information is available regarding the epidemiology, pathogenesis, and treatment [2]. Therefore, it is very important to examine the biological features of MBC in order to improve clinical outcome of the patients.

It is well known that estrogens contribute immensely to the development and/or progression of FBC. Concentration of biologically active estrogen estradiol is significantly high in FBC tissues, and it is locally produced from circulating inactive steroids by estrogen-producing enzymes, such as aromatase (conversion from circulating androstenedione to estrone or testosterone to estradiol), steroid sulfatase (STS; hydrolysis of circulating estrone sulfate to estrone), and 17β-hydroxysteroid dehydrogenase type 1 (17βHSD1; conversion of estrone to estradiol) [3]. Estrogen actions are initiated by binding of estrogens with estrogen receptors (i.e., ERα or ERβ), followed by transactivation of the target genes. Various estrogen-responsive genes have been identified in the breast carcinoma [4, 5], and analyses of these genes have greatly contributed to better understanding of molecular functions of estrogen actions in FBC [6]. The estrogen actions are considered to be mainly mediated through ERα in FBC [7, 8], and endocrine therapies, such as anti-estrogens (tamoxifen, etc.), aromatase inhibitors, and gonadotropin-releasing hormone (GnRH) agonists, are used in patients with ERα-positive FBC patients.

Estrogens are also speculated to play important roles in MBC, and tamoxifen is used in MBC patients as an endocrine therapy [9]. Various studies have demonstrated frequent expression of ERα in MBC tissues as well as ERβ and progesterone receptor (PR) [10–12], and immunolocalization of aromatase has been also reported in MBC [13]. However, intratumoral concentration of estrogens or expression of other estrogen-producing enzymes has not been reported in MBC. Moreover, no information is available regarding the expression profiles of estrogen-responsive genes in MBC, to the best of our knowledge. Therefore, it remains unclear whether estrogen actions and/or effectiveness of endocrine therapy in MBC could be the same as that in FBC.

Therefore, in this study, we examined intratumoral concentrations of estrogens, immunolocalization of estrogen-producing enzymes, and expression profiles of estrogen-induced genes in MBC tissues, and compared these findings with those in FBC, in order to examine the significance of estrogens in MBC.

Materials and Methods

Patients and Tissues

Two sets of tissue specimens were used in this study. The first set is composed of 14 snap-frozen specimens. Among these, four MBC tissues were obtained from patients who underwent surgical treatment from 2009 to 2010 at Tohoku University Hospital (Sendai, Japan), Tohoku Kosai Hospital (Sendai, Japan), Tohoku Rosai Hospital (Sendai, Japan), and Kansai Electric Power Hospital (Osaka, Japan). The mean age of these patients was 65 years (range, 62–67). Three non-neoplastic breast tissues were also collected from patients who underwent surgical treatment at Tohoku University Hospital, Tohoku Kosai Hospital, and Saitama Cancer center (Saitama, Japan; mean age, 65 years; range, 62–67 years), which were not matched with the carcinoma specimens. As a control group, seven specimens of FBC were obtained from postmenopausal patients who underwent surgical treatment from 2001 to 2003 at Tohoku University Hospital (mean age, 57 years; range, 50–69 years). These specimens were stored at −80 °C for subsequent hormone assays. Eight specimens of MBC and FBC were also used in microarray analysis.

The second set is composed of 102 specimens of breast carcinomas fixed in 10 % formalin and embedded in paraffin wax. Among these, 30 MBC tissues were obtained from patients who underwent surgical treatment from 1975 to 2010 at Tohoku University Hospital, Tohoku Kosai Hospital, Tohoku Rosai Hospital, Saitama Cancer Center, Sendai, and Kawasaki Medical School Hospital (Okayama, Japan). As a control group, we also used 72 FBC tissues collected from postmenopausal women who underwent surgical treatment from 1984 to 1992 at Tohoku University Hospital.

Research protocol was approved by Ethics Committee at Tohoku University School of Medicine.

Liquid Chromatography/Electrospray Tandem Mass Spectrometry (LC-MS/MS)

Concentrations of estradiol, estrone, testosterone, and androstenedione were measured by LC-MS/MS analysis in ASKA Pharma Medical Co., Ltd. (Kawasaki, Japan), as described previously [14, 15]. In the evaluation of estradiol concentration, we measured only 17β-estradiol, but not 17α-estradiol in this study. Briefly, tissue specimens were homogenized in 1 mL of distilled water, and steroid fraction was extracted with diethyl ether. In this study, we used an LC (Agilent 1100, Agilent Technologies, Waldbronn, Germany) coupled with an API 4000 triple-stage quadrupole

mass spectrometer (Applied Biosystems, Foster City, CA, USA) operated with electron spray ionization in the positive-ion mode, and the chromatographic separation was performed on Cadenza CD-C18 column (3×150 mm, 3.5 mm, Imtakt, Kyoto, Japan).

Laser-Capture Microdissection (LCM)/Microarray Analysis

Gene expression profiles of MBC and FBC cells were examined by microarray analysis. Four MBC and four FBC tissues were subjected to the study. LCM was conducted using the MMI Cellcut (Molecular Machines and Industries, Flughofstrase, Glattbrugg, Switzerland) according to previous reports [14, 16]. Briefly, breast carcinoma specimens (one specimen for each case) were embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetechnical Co., Tokyo, Japan), and serial sections were made at a thickness of 10 μm. Sections were stained with toluidine blue according to manufacturer's recommendation, and subsequently, breast carcinoma cells in each specimen (approximately 5,000 cells) were dissected under light microscopy and laser transferred from the serial sections. The total RNA (approximately 200 ng) was subsequently extracted from these cell fractions isolated by LCM using the RNeasy® Micro Kit (QIAGEN, Mannheim, Germany). Gene expression profiles were examined by microarray analyses. Whole Human Genome Oligo Microarray (G4112F, ID: 012391, Agilent Technologies), containing 41,000 unique probes, was used in this study, and sample preparation and processing were performed according to the manufacturer's protocol.

In our present study, we focused upon the expression profiles of two gene lists which were previously reported as estrogen-induced genes in FBC cell line MCF-7 [4, 5]. One was Frasor's list which consisted of 50 genes [4], and the other was Creighton's list which consisted of 63 genes [5]. If a gene was represented multiple times on the platform, the probe with strongest positive correlation with ESR1 (ERα) was selected. In order to compare the expression profiles of these genes, unsupervised hierarchical clustering analysis was performed using the Cluster and TreeView programs (the software copyright Stanford University 1998–1999, http://rana.stanford.edu) to generate tree structures based on the degree of similarity, as well as matrices comparing the levels of expression of individual genes in each specimens. Expression of genes was statistically evaluated by Student's t test, and $P<0.05$ was considered significant in this study.

Immunohistochemistry

The characteristics of primary antibody of aromatase [13], STS [17], and 17βHSD1 [15] were described previously. Monoclonal antibodies for ERα (ER1D5), ERβ (14C8), PR (MAB429), and Ki-67 (MIB1) were purchased from Immunotech (Marseille, France), Gene Tex (San Antonio, TX, USA), Chemicon (Temecula, CA, USA), and DAKO (Carpinteria, CA, USA), respectively. Rabbit polyclonal antibody for HER2 (A0485) was obtained from DAKO. Rabbit polyclonal antibody for receptor interacting protein 140 (RIP140) and retinoic acid receptor α (RARα) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

A Histofine Kit (Nichirei Biosciences, Tokyo, Japan), which employs the streptavidin-biotin amplification method, was used in this study. Immunoreactivity of estrogen-producing enzymes was detected in the cytoplasm, and the cases that had more than 10 % of positive cells were considered positive [18, 19]. Immunoreactivity of ERα, ERβ, PR, Ki-67, RIP140, and RARα was detected in the nucleus. These immunoreactivities were evaluated in more than 1,000 carcinoma cells, and subsequently, the percentage of immunoreactivity, i.e., labeling index (LI), was determined [20]. HER2 immunoreactivity was evaluated according to a grading system proposed in HercepTest (DAKO), and the cases with strongly circumscribed membrane staining of HER2 in more than 10 % carcinoma cells (i.e., score 3+) were considered positive in this study.

Results

Tissue Concentration of Estrogens and Androgens in MBC

We first examined tissue concentration of sex steroids in non-neoplastic male breast, MBC, and FBC tissues by LC-MS/MS. Median with minimum–max value of the estradiol level was 37.0 (8.0–74.0)pg/g in non-neoplastic male breast, 523 (267–633)pg/g in MBC, and 190 (15.7–540) pg/g in FBC (Fig. 1a). Tissue concentration of estradiol was significantly ($P=0.03$ and 14-fold) higher in MBC than non-neoplastic male breast tissues. Moreover, intratumoral estradiol concentration was 2.8-fold higher in MBC than in FBC tissues, although P value did not reach a significant level ($P=0.09$). On the other hand, tissue concentration of estrone was in 83.0 (56.0–359)pg/g in non-neoplastic male breast, 134 (67.0–280)pg/g in MBC, and 75.0 (13.0–555)pg/g in FBC, respectively, and the estrone level in MBC was not significantly different from that in non-neoplastic male breast or FBC ($P=0.72$ and $P=0.71$, respectively; Fig. 1b).

Tissue concentration of testosterone was high both in non-neoplastic male breast [1,519 (23.0–3,287)pg/g] and MBC [2,540 (1,454–3,483)pg/g], compared to that in FBC [133 (70.0–240)pg/g; $P=0.008$ in MBC vs. FBC], but no significant difference was detected between these two groups ($P=0.48$; Fig. 1c). Androstenedione has similar levels in these three groups [620 (53–7,525)pg/g in non-neoplastic male breast, 1,021 (291–1,805)pg/g in MBC, and 561 (160–5,785)pg/g in FBC] in this study (Fig. 1d).

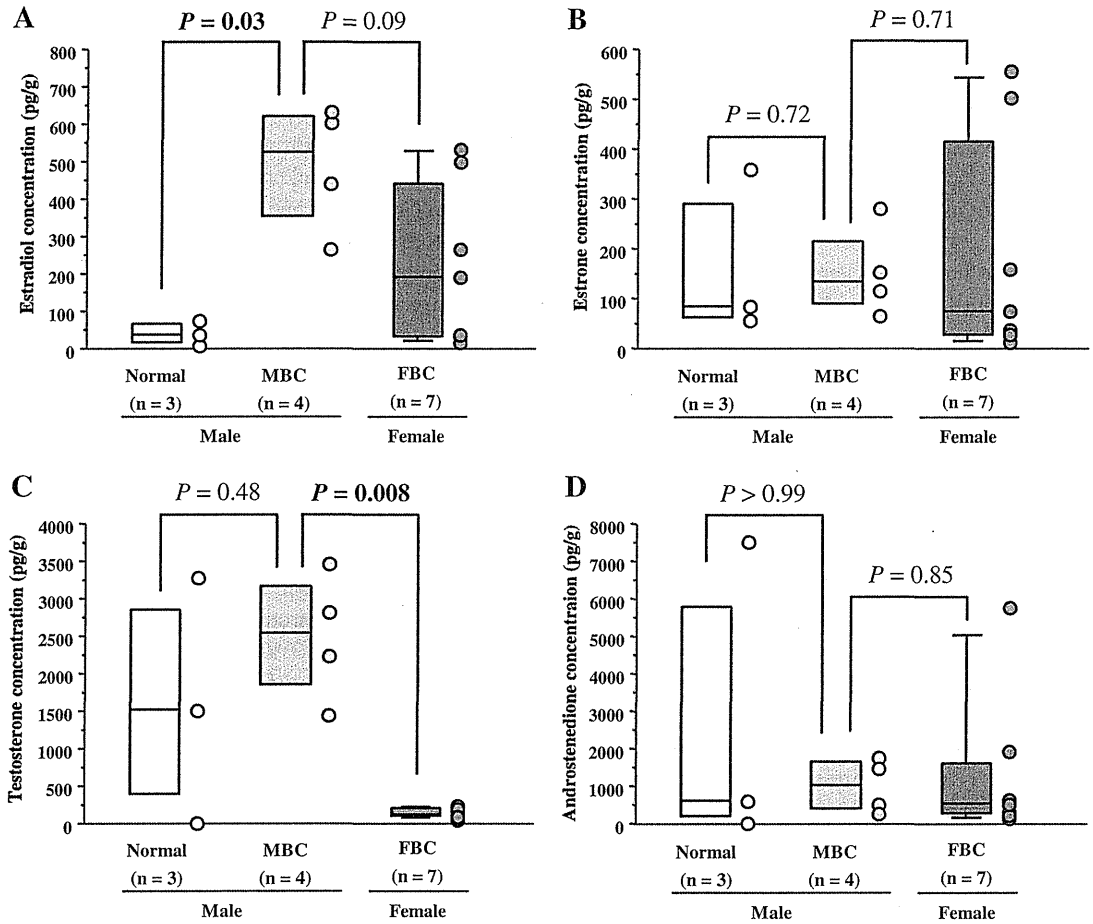

Fig. 1 Tissue concentration of estradiol (**a**), estrone (**b**), testosterone (**c**), and androstenedione (**d**) in non-neoplastic male breast, MBC, and FBC tissues. Each value was represented as a *circle*, and the grouped data were shown as *box-and-whisker plots*. The median value is demonstrated by a *horizontal line* in the box plot, and the *gray box* denotes the 75th (upper margin) and 25th percentiles of the values (lower margin). The *upper* and *lower bars* indicated the 90th and tenth percentiles, respectively. Statistical analysis was done by Mann–Whitney's *U* test; *P* values <0.05 were considered significant and indicated in *bold*

Expression Profiles of Estrogen-Induced Genes in MBC Compared with Those of FBC

We then performed microarray analysis in order to examine gene expression profiles of MBC cells isolated by LCM. Statistical analysis using Student's *t* test demonstrated that 12,295 probes showed significantly different expression between MBC and FBC cases. We then focused upon the expression profiles of two gene lists which were previously reported as estrogen-induced genes in FBC cell line MCF-7 (i.e., Frasor's list [4] and Creighton's list [5]) in order to examine molecular characteristics of estrogen actions in MBC. In the Frasor's list, 28 out of 50 (56 %) genes showed significantly different expression levels in MBC compared to FBC, and among these genes, 14 genes were highly expressed in MBC while 14 genes were lowly expressed (Table 1). In the Creighton's list, expression levels of 32 genes out of 63 (51 %) genes were significantly different between in MBC and FBC, and 18 genes were highly expressed in MBC while the other 14 genes were lowly expressed (Table 2). Five genes (RASGRP1, RARA, ADCY9, CXCL12, and NRIP1) were also included in these two gene lists, and expression levels of NRIP (P=0.0045) and ADCY9 (P=0.046) were significantly higher in MBC than FBC, and those of RARA (P=0.0012), RASGRP1 (P=0.011), and CXCL12 (P=0.012) were significantly lower in MBC.

As demonstrated in Fig. 2, results of unsupervised hierarchical cluster analysis revealed that MBC (n=4) and FBC cases (n=4) formed independent clusters regardless of the gene lists examined.

Immunolocalization of Estrogen-Producing Enzymes in MBC

We next immunolocalized estrogen-producing enzymes in 30 MBC tissues. Immunoreactivity of aromatase (Fig. 3a),

Table 1 List of genes identified as estrogen-induced genes by Frasor et al. (Frasor's list) [4]

Symbol	*P*	MBC vs. FBC	Symbol	*P*	MBC vs. FBC
CCND1	**0.041**	**L**	TGIF2	0.076	–
MYBL2	**0.027**	**L**	EGR3	0.36	–
RASGRP1[a]	**0.011**	**L**	**CXCL12**[a]	**0.012**	L
PKMYT1	0.13	–	GLRB	0.23	–
CBFA2T3	0.36	–	CHEK2	0.051	–
CDC20	**0.046**	**L**	FOS	0.056	–
IGFBP5	0.18	–	SLK	0.056	–
CCBP2	**0.0064**	**L**	**ELL2**	**<0.0001**	**H**
MYC	**0.015**	**L**	**RFC4**	**0.0084**	**H**
CCNA2	**0.0097**	**L**	**ADCY9**[a]	**0.046**	**H**
POLE2	**0.019**	**L**	**MYB**	**0.011**	**H**
BRCA2	**0.022**	**L**	**BIRC5**	**0.047**	**H**
RARA[a]	**0.0012**	**L**	**NRIP1**[a]	**0.0045**	**H**
HOXC5	**0.0043**	**L**	**MCM3**	**0.0021**	**H**
CALCR	**0.0023**	**L**	**RBBP7**	**0.0031**	**H**
POLA2	**0.011**	**L**	**RAB31**	**0.0022**	**H**
AREG	**0.0021**	**H**	WISP2	0.52	–
PCNA	**0.0093**	**H**	MCM2	0.52	–
OSTF1	**0.0039**	**H**	MCM5	0.31	–
GADD45B	**0.048**	**H**	CDC2	0.051	–
VEGF	0.27	–	AURKA	0.33	–
PPP2R1B	0.30	–	BUB1	0.76	–
STC2	**0.020**	**H**	TMF1	0.66	–
TSPAN5	0.088	–	CDC6	0.81	–
IGFBP4	0.12	–	JAK1	0.96	–

Comparison of gene expression between MBC and FBC was performed by Student's *t* test. $P < 0.05$ was considered positive and described as *boldface*

"H" means that the gene is highly expressed in MBC compared to FBC, and "L" means that the gene is lowly expressed in MBC compared to FBC

[a]Genes contained by both Frasor's and Creighton's lists

STS (Fig. 3b), and 17βHSD1 (Fig. 3c) was detected in the cytoplasm of carcinoma cells in MBC tissues, but STS immunoreactivity was weaker and focal. The number of positive cases was as follows: aromatase, 19/30 (63 %); STS, 2/30 (6.7 %); and 17βHSD1, 20/30 (67 %). Non-neoplastic mammary glands and intratumoral stroma were negative for aromatase (Fig. 3d), STS, and 17βHSD1 in this study.

Immunolocalization of ERs and Estrogen-Induced Genes in MBC Compared with FBC

We also evaluated an association of several immunohistochemical parameters between MBC (n=30) and FBC tissues (n=72). As shown in Table 3, ERα and ERβ LIs were significantly ($P<0.0001$ and $P=0.001$) higher in MBC than FBC. When cases with ER LI of 10 % were considered ER-positive breast carcinoma [17, 18], all MBC cases examined were positive for ERα, while 67 % (48/72) of FBC were positive for ERα. In addition, a great majority (77 %) of MBC cases showed double positive for ERα and ERβ, and its frequency was significantly ($P=0.0009$) higher than that in FBC (39 %). PR LI was also significantly ($P=0.011$) higher in MBC than FBC, and it was positively associated with ERα LI [$P=0.03$ and $r^2=0.16$ (data not shown)]. On the contrary, Ki67 LI was significantly ($P=0.019$) lower in MBC than FBC. HER2 status was not significantly different between these in this study.

Since our microarray analyses demonstrated different expression profiles of estrogen-induced genes in MBC from those in FBC (Fig. 2), we also performed immunohistochemistry for two representative genes included in both Frasor's and Creighton's lists [RARA (RARα) and NRIP1 (RIP140)] to confirm the results. RARα immunoreactivity was sporadically detected in the nuclei of MBC cells (Fig. 4a), and its LI was significantly ($P=0.0034$ and 0.62-fold) lower in MBC than FBC (Fig. 4b). On the other hand, RIP140 immunoreactivity was frequently detected in the nuclei of MBC cells (Fig. 4c), and RIP140 LI in MBC was significantly ($P=0.002$ and 1.91-fold) higher than FBC (Fig. 4d).

Discussion

To the best of our knowledge, this is the first study to have demonstrated intratumoral estrogen concentrations in MBC tissues. In the present study, tissue concentration of estradiol

Table 2 List of genes identified as estrogen-induced genes by Creighton et al. (Creighton's list) [5]

Symbol	*P*	MBC vs. FBC	Symbol	*P*	MBC vs. FBC
ATAD2	**0.0074**	**L**	PAK1IP1	0.61	–
CISH	0.056	–	CA12	0.80	–
GREB1	0.051	–	MYBL1	0.23	–
RASGRP1[a]	**0.011**	**L**	IRS1	0.37	–
ADSL	**0.0048**	**L**	KLF10	0.94	–
FLJ22624	**0.026**	**L**	**ADCY9**[a]	**0.046**	**H**
IGF1R	**0.015**	**L**	**FLJ11184**	**0.0064**	**H**
BRIP1	**0.0079**	**L**	**TIPARP**	**0.0045**	**H**
IL17RB	**0.0082**	**L**	TPBG	0.076	–
TEX14	**0.0004**	**L**	ZWILCH	0.25	–
PLK4	**0.012**	**L**	**MCM4**	**0.046**	**L**
RARA[a]	**0.0012**	**L**	**CXCL12**[a]	**0.012**	**L**
PTGES	0.066	–	**DSU**	**0.024**	**L**
SNX24	**0.016**	**L**	OLFM1	0.11	–
HSPB8	0.38	–	EEF1E1	0.43	–
TFF1	0.45	–	LOC56902	0.079	–
SIAH2	0.25	–	**NOL7**	**0.041**	**H**
OGFOD1	0.83	–	**SDCCAG3**	**0.030**	**H**
WDHD1	0.32	–	**PPIF**	**0.0046**	**H**
ZNF259	0.50	–	**MRPS2**	**0.024**	**H**
SLC39A8	0.83	–	**ALG8**	**0.0066**	**H**
WHSC1	0.63	–	**SLC9A3R1**	**0.014**	**H**
CTNNAL1	0.17	–	**XBP1**	**0.021**	**H**
DLEU1	0.18	–	CSPP1	0.76	–
FER1L3	**0.019**	**H**	THBS1	0.66	–
LRRC54	**0.024**	**H**	ENST00000379534	0.90	–
SGK3	**0.0068**	**H**	ENST00000278505	0.35	–
CTPS	**0.0059**	**H**	PPAT	0.61	–
LRP8	0.054	–	**MYB**	**0.029**	**H**
FHL2	**0.0005**	**H**	THRAP2	0.20	–
NRIP1[a]	**0.0045**	**H**	TPD52L1	0.57	–
DNAJC10	**0.042**	**H**			

Comparison of gene expression between MBC and FBC was performed by Student's *t* test. $P<0.05$ was considered positive and described as *boldface*

"H" means that the gene is highly expressed in MBC compared to FBC, and "L" means that the gene is lowly expressed in MBC compared to FBC

[a]Genes contained by both Frasor's and Creighton's lists

was significantly higher (14-fold) in MBC [523 (267–633) pg/g] than the non-neoplastic male breast tissues (Fig. 1a), whereas estrone, testosterone, and androstenedione levels did not significantly change between in these two groups (1.6-fold, 0.83-fold, and 1.6-fold, respectively). Serum estradiol concentration in men is known to be similar to that in postmenopausal women [21]. Chetrite et al. [22] previously showed that estradiol level was significantly higher in breast carcinomas in postmenopausal women [388±106 pg/g (mean±SEM)] than in the areas considered as morphologically normal in the same patients, which is currently explained by intratumoral production of estradiol [3]. Although serum estradiol level in MBC patients has been reported twofold higher than that in healthy subjects [23], our present results suggest possible local production of estradiol in MBC tissues as well as FBC.

In the breast carcinoma of postmenopausal women, intratumoral estradiol is produced by aromatase and/or STS pathways [24]. In our present study, aromatase immunoreactivity was detected in 63 % of MBC cases. Its frequency was in good consistent with a previous report [13], and similar to that in FBC reported previously (55–77 %) [25, 26]. The positivity of 17βHSD1 immunoreactivity in MBC in our present study (67 %) was also similar to previous reports in FBC (47–61 %) [27, 28]. On the other hand, STS immunoreactivity was detected only in 7 % of MBC cases in this study, which was much lower (approximately 0.1-fold) than that in FBC reported (60–90 %) [29, 30]. Therefore, it is suggested that estradiol is mainly synthesized by aromatase pathway in MBC rather than STS.

Results of our present study also showed that estradiol concentration was 2.8-fold higher in MBC than postmenopausal

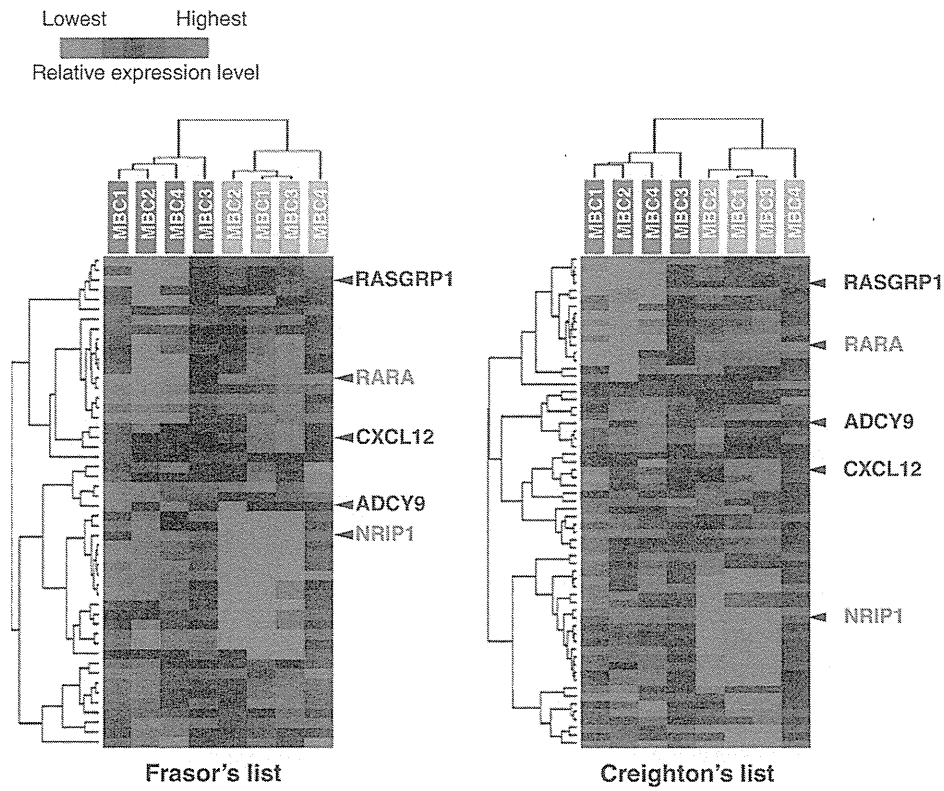

Fig. 2 Unsupervised hierarchical clustering analysis of mRNA expression levels focused on the genes which were previously reported as estrogen-induced genes [Frasor's list (*left*; 50 genes) and Creighton's list (*right*; 63 genes)]. Eight breast carcinoma samples [four MBCs (MBC1-4) and four FBCs (FBC1-4)] were used in this study, and genes and/or cases were grouped according to the similarity of gene expression, and the *shorter length of the branch* represents the higher similarity of cluster pairs. *Color of blocks* represents relative mRNA expression level of each gene, compared to the average in eight breast carcinoma samples. Five genes included in both lists (i.e., RASGRP1, RARA, ADCY9, CXCL12, and NRIP1) were indicated by *wedge*. Among these, two genes (RARA and NRIP1), which were subsequently evaluated by immunohistochemistry, were *highlighted in green*

FBC. Previously, Sonne-Hansen and Lykkesfeldt [31] reported that aromatase preferred testosterone as a substrate in MCF-7 breast carcinoma cells. In addition, plasma concentration of testosterone is approximately tenfold higher in men than postmenopausal women, while that of androstenedione is approximately1.5-fold

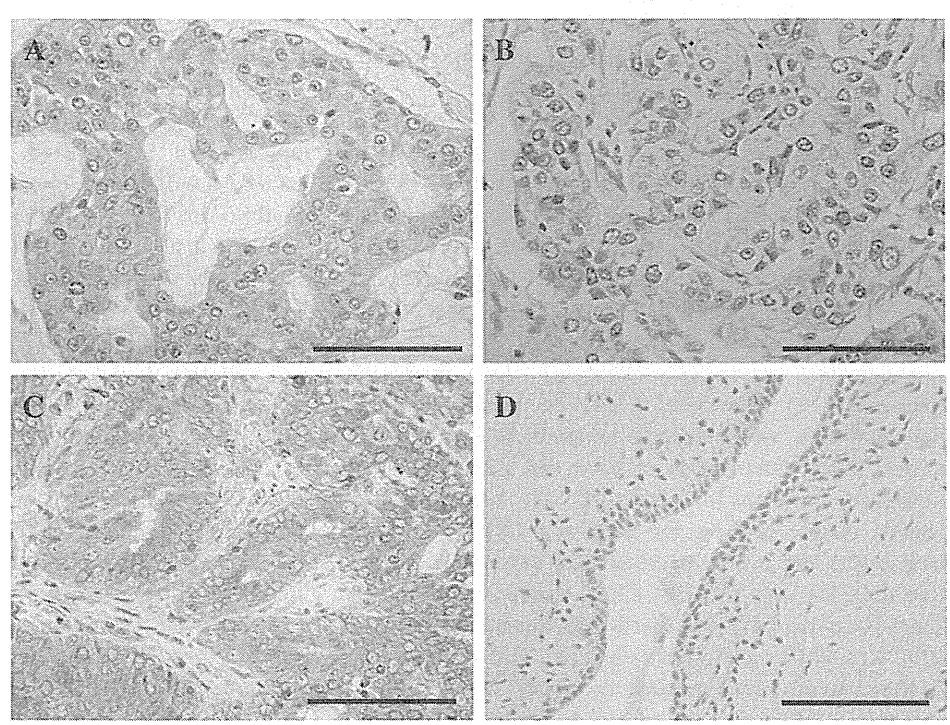

Fig. 3 Immunohistochemistry of estrogen-producing enzymes in MBC tissues. Immunoreactivity for aromatase (**a**), STS (**b**), and 17βHSD1 was visualized with 3,3′-diaminobenzidine (DAB; *brown*) and detected in the cytoplasm of carcinoma cells. Aromatase immunoreactivity was not detected in non-neoplastic mammary gland or stroma (**d**). *Bar*=100 μm, respectively

Table 3 Immunohistochemical features of MBC compared with FBC

	MBC n=30	FBC n=72	P value
ERα LI (%)[a]	90.5 (43–98.0)	40.0 (0.0–92)	**<0.0001**
ERα status			
Positive	30 (100 %)	48 (67 %)	
Negative	0 (0 %)	24 (33 %)	**<0.0001**
ERβ LI (%)[a]	27.5 (0–95)	8.5 (0–72)	**0.001**
ERβ status			
Positive	23 (77 %)	35 (49 %)	
Negative	7 (23 %)	37 (51 %)	**0.017**
ERα/ERβ status			
Positive/positive	23 (77 %)	28 (39 %)	
Others	7 (23 %)	44 (61 %)	**0.0009**
PR LI (%)[a]	43.5 (6–95)	17.5 (0–93)	**0.011**
HER2			
Positive	5 (17 %)	24 (33 %)	
Negative	25 (83 %)	48 (67 %)	0.099
Ki67 LI (%)[a]	15.5 (1.0–30)	20.0 (2.0–67)	**0.019**

[a] Data was presented as median with minimum–max or the number of cases with percentage. P value <0.05 was considered significant and described as *boldface*

higher in men [21]. Therefore, estradiol may be mainly produced from circulating testosterone by aromatase in MBC tissues. These findings also suggest that aromatase inhibitors are possibly effective in a selective group of MBC patients. A phase 2 trial used aromatase inhibitor, and GnRH analogue (SWOG-S 0511 trial) is currently ongoing in MBC patients [32].

The biological effects of estrogens are mediated through an initial interaction with ERα and/or ERβ, and ERs functions as hetero- or homodimers. In this study, both ERα and ERβ were more frequently immunolocalized in MBC than in FBC, which was in good agreement with previous reports [10–12]. Moreover, we also found that a great majority (77 %) of MBC cases showed double positive for ERα and ERβ, and its frequency was significantly (2.0-fold) higher than FBC cases (Table 1). Therefore, it may be possible to speculate that ERs are frequently heterodimerized in MBC tissues. Heterodimerization of ERα and ERβ modulates biological functions of each ER [33, 34], and FBC patients double positive for ERα and ERβ had longer disease-free and overall survival than those showed positive for ERα only [35, 36]. On the other hand, Weber-Chappuis et al. [37] suggested that functions of ER in MBC were different from that in FBC, and Johansson et al. [38] recently demonstrated that MBC was classified into two groups (i.e., luminal M1 and M2), those differed from the intrinsic subtypes of ER-positive FBC, by microarray analyses. Therefore, estrogen actions in MBC may not be necessarily the same as those in FBC, which is partly due to the different ERα/ERβ status from FBC.

Results of our microarray analysis did demonstrate that a majority of estrogen-induced genes (56 % in Frasor's list and 51 % in Creighton's list) showed significantly different expression between in MBC and FBC, and MBC cases formed a different cluster from FBC cases. We also confirmed these results by employing immunohistochemistry for representative genes (i.e., RARα and RIP140). Therefore, it is reasonably postulated that molecular functions of estrogens in MBC may be different from those in FBC based on the results above. However, it is also true that estrogen-induced genes examined in this study were identified in female breast cancer cell line MCF-7, and it is still not clarified whether these genes were similarly regulated by estrogen in MBC tissues or not, which also suggests that all the genes detected at markedly different levels in MBC compared to FBC were therefore not necessarily regulated by estrogens. In addition, only two genes on Creighton's list (CA12 and SIAH2) were included in the gene list, which was recently identified as MBC-specific genes by Johansson et al. [38]. Estrogen-induced genes are not determined yet in MBC because of unavailability of appropriate cell line and/or its relevant in vivo model. Therefore, further examinations are required to clarify the molecular features of estrogen actions in MBC.

Among the genes overexpressed in FBC (summarized in Tables 1 and 2), MYC (C-MYC) was well known to be associated with poor prognosis or adverse clinical outcome of ER-positive breast cancer patients [39], and RARA (RARα) upregulated 17βHSD1 and contributed to in situ production of estradiol in FBC [40]. IGF1R (insulin-like growth factor receptor) has been considered to promote breast carcinoma cell growth by interacting with estrogen signaling [41]. In addition, Ma et al. and Wang et al. independently reported that IL17RB (interleukin-17 receptor B) expression was significantly associated with increased risks of recurrence in ERα-positive breast cancer patients [42, 43]. However, among the genes highly expressed in MBC, MYB (c-myb) was associated with a good prognosis in the patients [44]. NRIP1 (RIP140) is a negative transcriptional regulator of hormone receptor [45, 46] and inhibited ERα activity in the breast carcinoma cells [43]. RBBP7 (RBAP46) also modulated estrogen responsiveness in breast carcinoma cells through an interaction with ERα [47] and inhibited an estrogen-stimulated progression of transformed breast epithelial

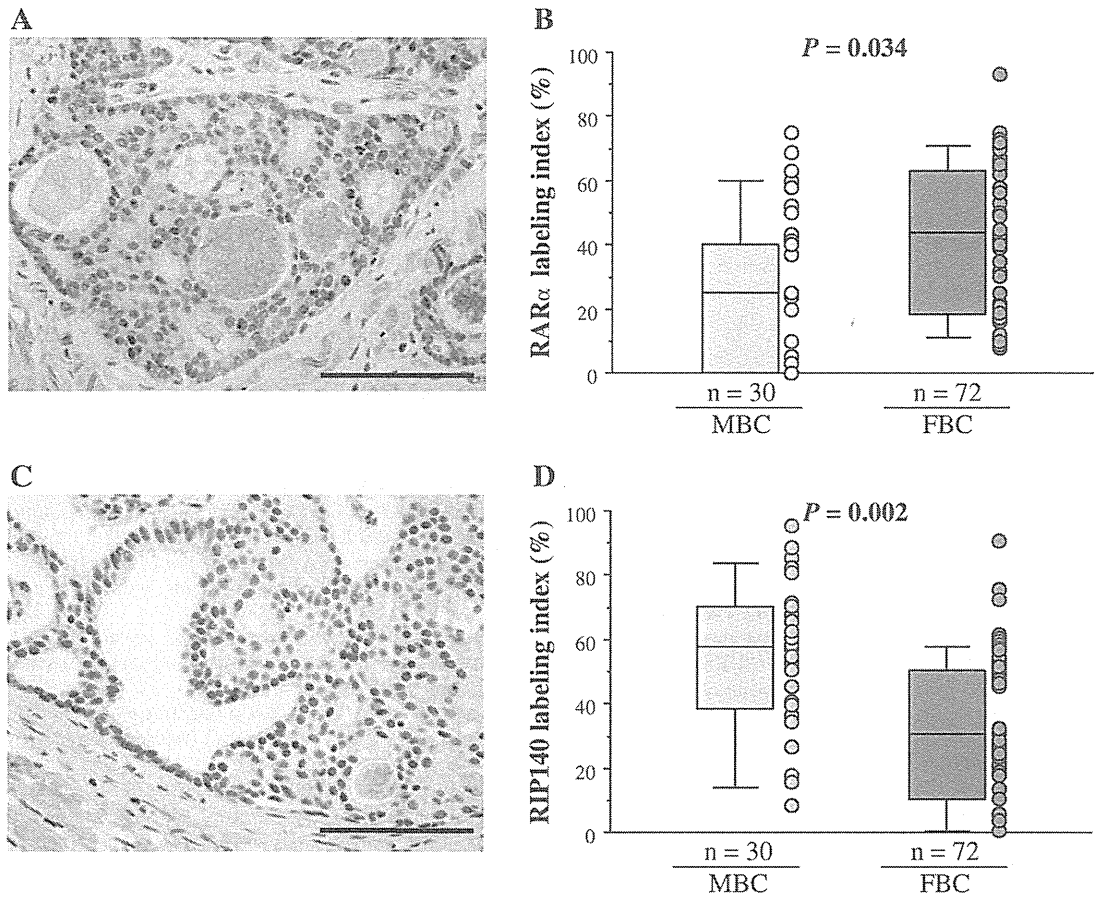

Fig. 4 Immunohistochemistry of RARα (**a**, **b**) and RIP140 (**c**, **d**) in MBC tissues. RARα (**a**) and RIP140 (**c**) immunoreactivity was visualized with DAB (*brown*) and detected in the nuclei of carcinoma cells. *Bar*=100 μm, respectively. Relative immunoreactivity of RARα and RIP140 was summarized in **b** and **d**, respectively. Each value was represented as a *circle*, and the grouped data were shown as *box-and-whisker plots*. The median value is demonstrated by a *horizontal line* in the box plot, and the *gray box* denotes the 75th (upper margin) and 25th percentiles of the values (lower margin). The *upper* and *lower bars* indicate the 90th and tenth percentiles, respectively. Statistical analysis was performed by Mann–Whitney's *U* test; *P* values <0.05 were considered significant and indicated in *bold*

cells [48]. In addition, FHL2 (four and a half LIM domains 2) was reported to inhibit proliferation and invasion of breast carcinoma cells by suppressing the function of ID3 (inhibitor of DNA binding 3), which was also known as one of the adverse prognostic factor of patients with breast cancer [49, 50]. Considering the functions of these gene above, estrogens may more efficiently promote aggressive clinical behavior in FBC than MBC, although some genes highly expressed in MBC were indeed associated with aggressive phenotypes of the breast carcinoma, such as AREG (amphiregulin) and XBP1 (X-box binding protein 1) [51, 52]. To date, tamoxifen is used as an endocrine therapy for MBC patients. However, it has been reported that expression profile of estrogen responsive gene was closely related to the response to tamoxifen in FBC patients [53]. Further examinations are required to clarify molecular functions of estrogen actions in MBC to improve the effectiveness of endocrine therapy for MBC patients.

In summary, intratumoral concentration of estradiol was significantly higher in MBC than non-neoplastic male breast tissues in this study, and aromatase and 17βHSD1 were frequently immunolocalized in MBC tissues. In addition, a great majority (77 %) of MBC cases showed positive for both ERα and ERβ, and its frequency was significantly higher than FBC cases. Results of microarray analysis revealed that expression profiles of genes known to be regulated by estrogen were markedly different between MBC and FBC. These results suggest that estradiol is mainly produced by aromatase from circulating testosterone in MBC tissues, and expression profiles of estrogen-induced genes in MBC are different from FBC, which may be partly due to their different ERα/ERβ status.

Acknowledgments We appreciate the skillful technical assistance of Mr. Katsuhiko Ono (Department of Anatomic Pathology, Tohoku University Graduate School of Medicine).

Disclosures The authors declare that there is no conflict of interest to be disclosed.

Funding This work was partly supported by Grant-in-Aid for Scientific Research (24790343) from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

References

1. Giordano SH, Cohen DS, Buzdar AU, Perkins G, Hortobagyi GN (2004) Breast carcinoma in men: a population-based study. Cancer 101:51–57
2. Nahleh Z, Girnius S (2006) Male breast cancer: a gender issue. Nat Clin Pract Oncol 3:428–437
3. Suzuki T, Miki Y, Nakamura Y et al (2005) Sex steroid-producing enzymes in human breast cancer. Endocr Relat Cancer 12:701–720
4. Frasor J, Danes JM, Komm B, Chang KC, Lyttle CR, Katzenellenbogen BS (2003) Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. Endocrinology 144:4562–4574
5. Creighton CJ, Cordero KE, Larios JM, Miller RS, Johnson MD, Chinnaiyan AM, Lippman ME, Rae JM (2006) Genes regulated by estrogen in breast tumor cells in vitro are similarly regulated in vivo in tumor xenografts and human breast tumors. Genome Biol 7 (4):R28, Epub 2006 Apr 7
6. Suzuki S, Takagi K, Miki Y et al (2012) Nucleobindin 2 in human breast carcinoma as a potent prognostic factor. Cancer Sci 103:136–143
7. Leygue E, Dotzlaw H, Watson PH, Murphy LC (1998) Altered estrogen receptor alpha and beta messenger RNA expression during human breast tumorigenesis. Cancer Res 58:3197–3201
8. Hayashi SI, Eguchi H, Tanimoto K et al (2003) The expression and function of estrogen receptor alpha and beta in human breast cancer and its clinical application. Endocr Relat Cancer 10:193–202
9. Cutuli B, Le-Nir CC, Serin D et al (2010) Male breast cancer. Evolution of treatment and prognostic factors. Analysis of 489 cases. Crit Rev Oncol Hematol 73:246–254
10. Rudlowski C, Friedrichs N, Faridi A et al (2004) Her-2/neu gene amplification and protein expression in primary male breast cancer. Breast Cancer Res Treat 84:215–223
11. Murphy CE, Carder PJ, Lansdown MR, Speirs V (2006) Steroid hormone receptor expression in male breast cancer. Eur J Surg Oncol 32:44–47
12. Shaaban AM, Ball GR, Brannan RA et al (2012) A comparative biomarker study of 514 matched cases of male and female breast cancer reveals gender-specific biological differences. Breast Cancer Res Treat 133:949–958
13. Sasano H, Kimura M, Shizawa S, Kimura N, Nagura H (1996) Aromatase and steroid receptors in gynecomastia and male breast carcinoma: an immunohistochemical study. J Clin Endocrinol Metab 81:3063–3067
14. Miki Y, Suzuki T, Tazawa C et al (2007) Aromatase localization in human breast cancer tissues: possible interactions between intratumoral stromal and parenchymal cells. Cancer Res 67:3945–3954
15. Takagi K, Miki Y, Nagasaki S et al (2010) Increased intratumoral androgens in human breast carcinoma following aromatase inhibitor exemestane treatment. Endocr Relat Cancer 17:415–430
16. Ebata A, Suzuki T, Takagi K et al (2012) Oestrogen-induced genes in ductal carcinoma in situ (DCIS): their comparison with invasive ductal carcinoma. Endocr Relat Cancer 19:485–496
17. Suzuki T, Miki Y, Nakata T et al (2003) Steroid sulfatase and estrogen sulfotransferase in normal human tissue and breast carcinoma. J Steroid Biochem Mol Biol 86:449–454
18. Penning TM, Steckelbroeck S, Bauman DR et al (2006) Aldo-keto reductase (AKR) 1C3: role in prostate disease and the development of specific inhibitors. Mol Cell Endocrinol 248:182–191
19. Suzuki T, Miki Y, Moriya T et al (2007) 5Alpha-reductase type 1 and aromatase in breast carcinoma as regulators of in situ androgen production. Int J Cancer 120:285–291
20. Ishibashi H, Suzuki T, Suzuki S et al (2005) Progesterone receptor in non-small cell lung cancer—a potent prognostic factor and possible target for endocrine therapy. Cancer Res 65:6450–6458
21. Greenspan FS, Strewler GJ (1997) Basic & clinical endocrinology. Appleton Lange, Stamford
22. Chetrite GS, Cortes-Prieto J, Philippe JC, Wright F, Pasqualini JR (2000) Comparison of estrogen concentrations, estrone sulfatase and aromatase activities in normal, and in cancerous, human breast tissues. J Steroid Biochem Mol Biol 72:23–27
23. Nirmul D, Pegoraro RJ, Jialal I, Naidoo C, Joubert SM (1983) The sex hormone profile of male patients with breast cancer. Br J Cancer 48:423–427
24. 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
25. Ellis MJ, Miller WR, Tao Y et al (2009) Aromatase expression and outcomes in the P024 neoadjuvant endocrine therapy trial. Breast Cancer Res Treat 116:371–378
26. Geisler J, Suzuki T, Helle H et al (2010) Breast cancer aromatase expression evaluated by the novel antibody 677: correlations to intra-tumor estrogen levels and hormone receptor status. J Steroid Biochem Mol Biol 118:237–241
27. Poutanen M, Isomaa V, Lehto VP, Vihko R (1992) Immunological analysis of 17 beta-hydroxysteroid dehydrogenase in benign and malignant human breast tissue. Int J Cancer 50:386–390
28. Suzuki T, Moriya T, Ariga N, Kaneko C, Kanazawa M, Sasano H (2000) 17Beta-hydroxysteroid dehydrogenase type 1 and type 2 in human breast carcinoma: a correlation to clinicopathological parameters. Br J Cancer 82:518–523
29. Yamamoto Y, Yamashita J, Toi M et al (2003) Immunohistochemical analysis of estrone sulfatase and aromatase in human breast cancer tissues. Oncol Rep 10:791–796
30. Tsunoda Y, Shimizu Y, Tsunoda A, Takimoto M, Sakamoto MA, Kusano M (2006) Steroid sulfatase in breast carcinoma and change of serum estrogens levels after operation. Acta Oncol 45:584–589
31. Sonne-Hansen K, Lykkesfeldt AE (2005) Endogenous aromatization of testosterone results in growth stimulation of the human MCF-7 breast cancer cell line. J Steroid Biochem Mol Biol 93:25–34
32. Korde LA, Zujewski JA, Kamin L et al (2010) Multidisciplinary meeting on male breast cancer: summary and research recommendations. J Clin Oncol 28:2114–2122
33. Gustafsson JA (2006) ERbeta scientific visions translate to clinical uses. Climacteric 9:156–160
34. Williams C, Edvardsson K, Lewandowski SA, Ström A, Gustafsson JA (2008) A genome-wide study of the repressive effects of estrogen receptor beta on estrogen receptor alpha signaling in breast cancer cells. Oncogene 27:1019–1032
35. Nakopoulou L, Lazaris AC, Panayotopoulou EG et al (2004) The favourable prognostic value of oestrogen receptor beta immunohistochemical expression in breast cancer. J Clin Pathol 57:523–528
36. Honma N, Horii R, Iwase T et al (2005) Clinical importance of estrogen receptor-beta evaluation in breast cancer patients treated with adjuvant tamoxifen therapy. J Clin Oncol 26:3727–3734

37. Weber-Chappuis K, Bieri-Burger S, Hurlimann J (1996) Comparison of prognostic markers detected by immunohistochemistry in male and female breast carcinomas. Eur J Cancer 32A:1686–1692
38. Johansson I, Nilsson C, Berglund P et al (2012) Gene expression profiling of primary male breast cancers reveals two unique subgroups and identifies N-acetyltransferase-1 (NAT1) as a novel prognostic biomarker. Breast Cancer Res 14:R31
39. Chen Y, Olopade OI (2008) MYC in breast tumor progression. Expert Rev Anticancer Ther 8:1689–1698
40. Suzuki T, Moriya T, Sugawara A, Ariga N, Takabayashi H, Sasano H (2001) Retinoid receptors in human breast carcinoma: possible modulators of in situ estrogen metabolism. Breast Cancer Res Treat 65:31–40
41. Gaben AM, Sabbah M, Redeuilh G, Bedin M, Mester J (2012) Ligand-free estrogen receptor activity complements IGF1R to induce the proliferation of the MCF-7 breast cancer cells. BMC Cancer 12:291
42. Ma XJ, Wang Z, Ryan PD et al (2004) A two-gene expression ratio predicts clinical outcome in breast cancer patients treated with tamoxifen. Cancer Cell 5:607–616
43. Wang Z, Dahiya S, Provencher H et al (2007) The prognostic biomarkers HOXB13, IL17BR, and CHDH are regulated by estrogen in breast cancer. Clin Cancer Res 13:6327–6334
44. Guérin M, Sheng ZM, Andrieu N, Riou G (1990) Strong association between c-myb and oestrogen-receptor expression in human breast cancer. Oncogene 5:131–135
45. Cavaillès V, Dauvois S, L'Horset F et al (1995) Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor. EMBO J 14:3741–3751
46. Augereau P, Badia E, Balaguer P et al (2006) Negative regulation of hormone signaling by RIP140. J Steroid Biochem Mol Biol 102:51–59
47. Creekmore AL, Walt KA, Schultz-Norton JR et al (2008) The role of retinoblastoma-associated proteins 46 and 48 in estrogen receptor alpha mediated gene expression. Mol Cell Endocrinol 291:79–86
48. Zhang TF, Yu SQ, Wang ZY (2007) RbAp46 inhibits estrogen-stimulated progression of neoplastigenic breast epithelial cells. Anticancer Res 27:3205–3209
49. Chen YH, Wu ZQ, Zhao YL et al (2012) FHL2 inhibits the Id3-promoted proliferation and invasive growth of human MCF-7 breast cancer cells. Chin Med J Engl 125:2329–2333
50. Gupta GP, Perk J, Acharyya S et al (2007) ID genes mediate tumor reinitiation during breast cancer lung metastasis. Proc Natl Acad Sci U S A 104:19506–19511
51. Busser B, Sancey L, Brambilla E, Coll JL, Hurbin A (2011) The multiple roles of amphiregulin in human cancer. Biochim Biophys Acta 1816:119–131
52. Sengupta S, Sharma CG, Jordan VC (2010) Estrogen regulation of X-box binding protein-1 and its role in estrogen induced growth of breast and endometrial cancer cells. Horm Mol Biol Clin Investig 2:235–243
53. Oh DS, Troester MA, Usary J et al (2006) Estrogen-regulated genes predict survival in hormone receptor-positive breast cancers. J Clin Oncol 24:1656–1664

Breast Cancer Res Treat (2012) 134:661–670
DOI 10.1007/s10549-012-2109-2

PRECLINICAL STUDY

Predictions of the pathological response to neoadjuvant chemotherapy in patients with primary breast cancer using a data mining technique

M. Takada · M. Sugimoto · S. Ohno · K. Kuroi · N. Sato · H. Bando · N. Masuda · H. Iwata · M. Kondo · H. Sasano · L. W. C. Chow · T. Inamoto · Y. Naito · M. Tomita · M. Toi

Received: 21 March 2012 / Accepted: 22 May 2012 / Published online: 12 June 2012

Abstract Nomogram, a standard technique that utilizes multiple characteristics to predict efficacy of treatment and likelihood of a specific status of an individual patient, has been used for prediction of response to neoadjuvant chemotherapy (NAC) in breast cancer patients. The aim of this study was to develop a novel computational technique to predict the pathological complete response (pCR) to NAC in primary breast cancer patients. A mathematical model using alternating decision trees, an epigone of decision tree, was developed using 28 clinicopathological variables that were retrospectively collected from patients treated with NAC ($n = 150$), and validated using an independent dataset from a randomized controlled trial ($n = 173$). The model selected 15 variables to predict the pCR with yielding area under the receiver operating characteristics curve (AUC) values of 0.766 [95 % confidence interval (CI)], 0.671–0.861, P value < 0.0001) in cross-validation using training dataset and 0.787 (95 % CI 0.716–0.858, P value < 0.0001) in the validation dataset. Among three subtypes of breast cancer, the luminal subgroup showed the best discrimination (AUC = 0.779, 95 % CI 0.641–0.917, P value = 0.0059). The developed model (AUC = 0.805, 95 % CI 0.716–0.894, P value < 0.0001) outperformed multivariate logistic regression (AUC = 0.754, 95 % CI 0.651–0.858, P value = 0.00019) of validation datasets without missing values ($n = 127$). Several analyses, e.g.

Electronic supplementary material The online version of this article (doi:10.1007/s10549-012-2109-2) contains supplementary material, which is available to authorized users.

M. Takada · M. Toi (✉)
Department of Breast Surgery, Graduate School of Medicine, Kyoto University, 54 Kawaracho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan
e-mail: toi@kuhp.kyoto-u.ac.jp

M. Takada
Japan Society for the Promotion of Science, Tokyo, Japan

M. Sugimoto
Medical Innovation Centre, Kyoto University Graduate School of Medicine, Kyoto University, Kyoto, Japan

M. Sugimoto · Y. Naito · M. Tomita
Institute for Advanced Biosciences, Keio University, Tsuruoka, Yamagata, Japan

S. Ohno
Department of Breast Oncology, National Kyushu Cancer Centre, Fukuoka, Japan

K. Kuroi
Department of Surgery, Tokyo Metropolitan Cancer and Infectious Diseases Centre, Komagome Hospital, Tokyo, Japan

N. Sato
Department of Surgery, Niigata Cancer Centre Hospital, Niigata, Japan

H. Bando
Department of Breast and Endocrine Surgery, Faculty of Medicine, University of Tsukuba, Tsukuba, Ibaraki, Japan

N. Masuda
Department of Surgery, Breast Oncology, Osaka National Hospital, Osaka, Japan

H. Iwata
Department of Breast Oncology, Aichi Cancer Centre, Nagoya, Aichi, Japan

M. Kondo
Department of Health Care Policy and Management, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan

H. Sasano
Department of Pathology, Tohoku University Hospital and School of Medicine, Sendai, Miyagi, Japan

bootstrap analysis, revealed that the developed model was insensitive to missing values and also tolerant to distribution bias among the datasets. Our model based on clinicopathological variables showed high predictive ability for pCR. This model might improve the prediction of the response to NAC in primary breast cancer patients.

Keywords Breast cancer · Data mining · Neoadjuvant chemotherapy · Nomogram · Prediction model

Introduction

Neoadjuvant chemotherapy (NAC) is the administration of chemotherapy before surgical treatment of cancer. The clinical advantages of NAC include tumour size reduction, which improves the breast conservation rate, and determination of chemosensitivity to help design later adjuvant therapy [1]. Several meta-analyses have revealed that patients with pathological complete response (pCR) after NAC showed higher survival rates than those without pCR [1–4], indicating that pCR might represent a surrogate prognostic indicator in these patients. Thus, predicting pCR using information collected before NAC has been proposed, with the most commonly used predictive factors including oestrogen receptor (ER) status, human epidermal growth factor receptor 2 (HER2/neu) status, histological grade and proliferative activity [5–7]. Recent studies showed that the sensitivity to chemotherapy differs according to cancer phenotype classified mainly by ER and HER2 status [8–10]. Luminal A subtype (ER-positive, HER2-negative and low-grade or low-proliferative phenotype) exhibited lower sensitivity to chemotherapy despite better prognosis than other phenotypes, and hormonal therapy alone is the preferred treatment for this subtype [11].

Nomograms, which integrate clinical and pathological variables using multiple logistic regression (MLR), have been developed and are well validated to predict pCR after NAC [12, 13]. These tools are now widely used by oncologists via sophisticated web interfaces. In comparative studies using prospective cohorts, nomograms showed similar performance to molecular tests designed to predict pCR following NAC [14, 15]. The gene signatures to predict prognosis were also expected to predict chemosensitivity [16–19], although the predictive ability was limited in those studies. Thus, new prediction tools, even with the use of molecular/clinicopathological factors, are now needed.

MLR has several limitations. First, it can deal with only few independent variables to avoid over-fitting to the given datasets. Second, MLR is sensitive to missing values, a frequent occurrence in retrospectively collected data. Third, MLR cannot tolerate the distribution bias of variables among multiple datasets (usually obtained from different institutes), which reduces its generalizability. Thus, we used a data mining technique to address the following problems: (1) limits in the number of variables that can be included in a model, (2) missing values and (3) bias among datasets. Using alternating decision tree (ADTree), an accurate and versatile decision-tree type data mining method [20], we developed and validated a mathematical model to predict pCR after NAC in patients with primary breast cancer.

Materials and methods

Participants and treatments

The study protocol was approved by the institutional review board of Kyoto University Hospital. We disclosed the details of the study to all of the participants in lieu of obtaining informed consent because the Japanese ethics guidelines for epidemiologic research allow observational studies to use anonymous clinical data after disclosing the study details to the potential participants.

We included patients who had participated in the Organisation for Oncology and Translational Research (OOTR) N003 trial. This was a randomized trial of patients with operable breast cancer treated with docetaxel with or without capecitabine after four cycles of NAC consisting of 5-fluorouracil, epirubicin and cyclophosphamide (FEC) (UMIN ID: C000000322, http://www.umin.ac.jp/ctr/index.htm). Patients who received the same chemotherapy regimen in regular clinical practice were also included in this study. Only patients with a tumour size of $\leq$5 cm and who had completed $\geq$75 % of the planned courses of NAC were included.

First, we conducted an exploratory analysis using a dataset of 58 patients collected consecutively from Tokyo Metropolitan Cancer and Infectious Diseases Centre

L. W. C. Chow
Comprehensive Centre for Breast Diseases, UNIMED Medical Institute, Hong Kong, China

T. Inamoto
Department of Breast Surgery, Tenri Hospital, Tenri, Nara, Japan

Y. Naito · M. Tomita
Faculty of Environment and Information Studies, Keio University, Fujisawa, Kanagawa, Japan

Y. Naito · M. Tomita
Graduate School of Media and Governance, Keio University, Fujisawa, Kanagawa, Japan

Komagome Hospital. Additional patients were then collected from Osaka National Hospital and Tsukuba University Hospital to develop the prediction model. The training dataset consisted of 150 patients treated at the three institutions from 2005 to 2009. This included 89 patients who participated in the OOTR N003 trial. The newly developed prediction model was applied to an external validation dataset consisting of 173 patients from the OOTR N003 trial. This validation dataset was obtained from three randomly selected institutions that had participated in the OOTR N003 trial (Niigata Cancer Centre Hospital, National Kyushu Cancer Centre and Aichi Cancer Centre).

All of the patients included in this study received the same treatment protocol, consisting of four courses of FEC (5-fluorouracil 500 mg/m^2, epirubicin 100 mg/m^2 and cyclophosphamide 500 mg/m^2, i.v., every 3 weeks) followed by four courses of docetaxel (75 mg/m^2, i.v., every 3 weeks) with or without capecitabine (1,650 mg/m^2/day, oral administration, for 14 days every 3 weeks).

Data collection

Data for 28 clinicopathological variables were retrospectively collected from databases maintained at each institution (Table 1). All of the mammography and ultrasonography images were reviewed by physicians certified in imaging diagnosis by the relevant accreditation organizations in Japan. The Response Evaluation Criteria in Solid Tumours (RECIST) were used to classify the clinical response to NAC. According to the protocol of the OOTR N003 trial, the clinical response was evaluated after both the FEC treatment (i.e. the clinical response after anthracycline) and the taxane-containing regimen (i.e. the clinical response after taxane). Information pertaining to histological type, ER status, progesterone receptor (PgR) status, HER2 status and histological/nuclear grade of needle biopsy specimens were collected from the original pathology report of each patient. At each institution, the surgical specimen obtained following NAC was serially sectioned, stained with haematoxylin and eosin (H&E) and diagnosed by experienced pathologists. pCR was defined as the absence of residual invasive cancer cells in the breast and the axillary lymph nodes (ypT0/is + ypN0).

Data analysis

For statistical analyses, we quantitatively graded the variables using the criteria established by a committee of specialists from the fields of breast surgery, diagnostic radiology and pathology.

The ADTree model was developed using the training dataset and validated using the independent validation

Table 1 Characteristics of patients in the training and validation datasets

Variables	Training dataset		Validation dataset		P value[d]
	n	%	*n*	%	
No. of patients	150		173		
Age (years)					
Median	50		48		0.131
Range	(27–71)		(29–68)		
Body mass index (kg/m^2)					
Median	22.7		21.2		0.0004
Range	(16.9–35.8)		(15.6–43)		
Menopausal status					
Pre-menopause	75	50.0	105	60.7	0.054
Post-menopause	75	50.0	68	39.3	
Physical examination					
Palpable lump					
Yes	149	99.3	166	96.0	0.136
No	1	0.7	5	2.9	
Unknown	0	0	2	1.2	
Skin dimpling					
Yes	31	20.7	28	16.2	0.281
No	116	77.3	143	82.7	
Unknown	3	2.0	2	1.2	
Mammography					
Presence of a mass					
Yes	92	61.3	113	65.3	0.102
Focal asymmetry	19	12.7	35	20.2	
No	26	17.3	20	11.6	
Unknown	13	8.7	5	2.9	
Presence of calcifications					
Yes	45	30.0	80	46.2	0.035
No	87	58.0	88	50.9	
Unknown	15	10.0	5	2.9	
Architectural distortion					
Yes	27	18.0	22	12.7	0.110
No	108	72.0	145	83.8	
Unknown	15	10.0	6	3.5	
Ultrasonography					
Presence of masses					
Yes	148	98.7	172	99.4	0.480
No	2	1.3	1	0.6	
Maximum tumour size (mm)					
Median	26		29		0.012
Range	(11–48)		(11–49)		
Unknown	0	0	8	4.7	
Depth/width ratio					
Median	0.67		0.58		<0.0001
Range	(0.23–2.06)		(0.22–1.18)		

Table 1 continued

Variables	Training dataset		Validation dataset		*P* value[d]
	n	%	*n*	%	
Unknown	3	2.0	21	12.2	
Echogenic halo					
Yes	68	45.9	56	32.6	0.026
No	79	53.4	109	63.4	
Unknown	1	0.7	7	4.1	
Posterior acoustic features					
Enhancement	33	22.3	61	35.5	0.024
None	75	50.7	66	38.4	
Shadowing	37	25.0	41	23.8	
Unknown	3	2.0	4	2.3	
Interruption of the anterior border of the mammary gland					
Yes	123	83.1	146	84.9	0.694
No	22	14.9	23	13.4	
Unknown	3	2.0	3	1.7	
Histological type					
Invasive ductal carcinoma	146	97.3	170	98.3	0.566
Invasive lobular carcinoma	4	2.7	3	1.7	
ER status[a]					
Positive	105	70.0	102	59	0.024
Negative	42	28.0	70	40.5	
Unknown	3	2.0	1	0.6	
PgR status[a]					
Positive	69	46.0	84	48.6	0.735
Negative	78	52.0	88	50.9	
Unknown	3	2.0	1	0.6	
HER2 status[b]					
Positive	19	12.7	38	22.0	0.026
Negative	125	83.3	127	73.4	
Unknown	6	4.0	8	4.6	
Triple-negative phenotype[c]					
Yes	31	20.7	44	25.4	0.293
No	113	75.3	121	70.0	
Unknown	6	4.0	8	4.6	
Histological/nuclear grade					
1/2	94	62.7	89	51.4	0.446
3	49	32.7	38	22.0	
Unknown	7	4.7	46	26.6	
Mitotic index					
1	68	45.3	68	39.3	0.137
2	40	26.7	23	13.3	
3	33	22.0	21	12.1	
Unknown	9	6.0	61	35.3	
Treatment regimen					
FEC-T	108	72.0	94	54.3	0.001
FEC-TX	42	28.0	79	45.7	

NS not significant, *NC* not collected, *FEC* 5-fluorouracil + epirubicin + cyclophosphamide, *T* taxane, *TX* taxane + capecitabine

[a] ER-positive or PgR-positive was defined as 10 % or more of cells with positive staining or Allred score of 3 or more

[b] HER2-positive was defined as a score of 3+ on immunohistochemical testing or a positive score on fluorescence in situ hybridization testing

[c] Triple negative was defined as ER, PgR and HER2 negative

[d] χ^2 test, Mann–Whitney *U* or *t* test

dataset. To enhance model accuracy, we used ensemble methods: multiple ADTree models were developed and the mean prediction of these models was used as the final prediction [21]. The model was optimized by cross-validation (CV) and the area under the receiver operating characteristics curve (AUC) for discriminating pCR from non-pCR was determined.

The importance of variables in the ADTree model was evaluated based on the decrease in prediction accuracy (AUC values) by replacing the actual value with a random value for each variable (sensitivity analysis). To evaluate the significance of missing values in the developed model, the missing values were replaced with random values and the decrease in AUC value was assessed (missing value analysis). The prediction accuracy was evaluated using a smaller number of ADTrees in the developed model than the optimized number (pruning analysis). To elucidate the relationship between generalizability and variable distribution bias between the training and the validation dataset, we integrated all of the data and randomly split it into two datasets. The ADTree model was developed using one of these datasets and validated using the other dataset (random split analysis). Each analysis was repeated 200 times with different random values.

We also developed an MLR model using the training dataset. Details of this model and the software used are described in the Supplementary Materials and methods.

Results

The clinicopathological variables of each dataset are summarized in Table 1. The training dataset included more ER-positive or HER2-negative patients compared with the validation dataset (*P* value = 0.024 and 0.026, respectively). Overall, 16 % of patients in the training dataset and 22.5 % in the validation dataset achieved pCR

Table 2 Treatment outcomes of the training and validation datasets

Outcomes	Training dataset		Validation dataset		p value[a]
	n	%	n	%	
No. of patients	150		173		
Clinical response after anthracycline treatment					
CR + PR	99	66.0	151	87.3	<0.0001
SD	50	33.3	21	12.1	
PD	0	0	1	0.6	
Unknown	1	0.7	0	0	
Clinical response after taxane treatment					
CR + PR	128	85.3	164	94.8	0.006
SD	21	14	7	4	
PD	1	0.7	2	1.2	
Breast surgery					
Mastectomy	32	21.3	53	30.6	0.058
Breast-conserving surgery	118	78.7	120	69.4	
pCR (ypT0/is + ypN0)					
Yes	24	16.0	39	22.5	0.139
No	126	84.0	134	77.5	

CR complete response, *PR* partial response, *SD* stable disease, *PD* progression disease

[a] χ^2 test

(P value = 0.139) (Table 2). The rates of pCR and breast conservation were not significantly different between the institutions (P value = 0.06 and 0.30, respectively). The clinical responses after anthracycline and taxane, however, were significantly lower in the training dataset than in the validation dataset (P value < 0.0001 and P = 0.006, respectively).

The selected model showing the best AUC value in the CVs contained 19 ADTrees with three variables on each tree (Fig. 1a; Supplementary Fig. S1). In total, 15 variables were included: three general [body mass index (BMI), menopausal status and the presence of skin dimpling], five ultrasonographic (maximum tumour size, tumour depth/width ratio, echogenic halo, interruption of the anterior border of the mammary gland and posterior acoustic features), three mammographic (the presence of calcifications, the presence of a mass and architectural distortion), and four pathological variables (mitotic index and the status of ER, PgR and HER2). The method used to calculate the probability of pCR using this model is shown in Fig. 1b and Supplementary Fig. S2.

The receiver operating characteristics (ROC) curves and the dot-plots of the pCR for each dataset are shown in Fig. 2. The AUC values were 0.766 (95 % CI 0.671–0.861, P value < 0.0001) in the CV using the training dataset and 0.787 (95 % CI 0.716–0.858, P value < 0.0001) using the validation dataset. The model could discriminate pCR from non-pCR patients at significant levels in both the training and the validation datasets (P value < 0.0001). When the threshold for a low risk of pCR was defined as 20 % for example, the false-negative rate was 7.7 % and the negative predictive value was 95.9 % using the validation dataset. The AUC of bootstrap analysis (200 repetitions) performed to obtain unbiased estimates was 0.791 (95 % CI: 0.786–0.796) using the validation dataset.

To assess the prediction ability by integrating early clinical response, we developed a MLR model comprising two variables; the predicted probability of pCR determined by ADTree and the clinical responses after anthracycline or taxane treatment. The accuracy of the ADTree model was enhanced by including the clinical response to NAC (Supplementary Fig. S3). The AUC values for the validation datasets were 0.820 (95 % CI 0.757–0.883, P value < 0.0001) and 0.855 (95 % CI 0.794–0.916, P value < 0.0001) after including the clinical responses after anthracycline and taxane treatment, respectively.

We evaluated the discriminative ability of our model in three subgroups of patients with luminal (ER-positive and any HER2 status; n = 102), HER2-positive (ER-negative and HER2-positive; n = 24) or triple-negative (ER- and HER2-negative; n = 44) patterns of receptor expression. The model showed significant discrimination of the luminal subgroup (P value = 0.0059), poor discrimination of the triple-negative subgroup (P value = 0.743) and moderate discrimination of the HER2-positive subgroup (P value = 0.074) (Fig. 3).

The sensitivity analysis revealed large decreases in AUC values when ER, PgR, HER2 and echogenic halo were randomly replaced, indicating high importance of these factors. On the other hand, the AUC values were hardly changed when imaging findings were randomly replaced, indicating little importance of these parameters (Fig. 4a). In missing value analysis, the median AUC was 0.786 (95 % CI 0.785–0.787) using validation datasets including patients with at least one missing value (n = 121). As another approach to evaluate the importance of the variables included in the model, pruning analysis was performed to reduce the number of trees, which also reduces the number of variables. In this analysis, the AUC value for the overall dataset remained high (>0.78) when the number of trees was >4 and the number of variables was >6 (Fig. 4b). When the number of variables was reduced to 8 for example, discrimination of ER-positive and, particularly, HER2-positive subtypes deteriorated (P value = 0.0266 and P value = 0.725), respectively (Supplementary Fig. S4). In random split analysis, the median AUC value was 0.776 (95 % CI 0.773–0.776), almost identical to the AUC value obtained using the original validation dataset (0.787).

To compare ADTree and MLR, a MLR model was developed using our training dataset and consisted of ER,

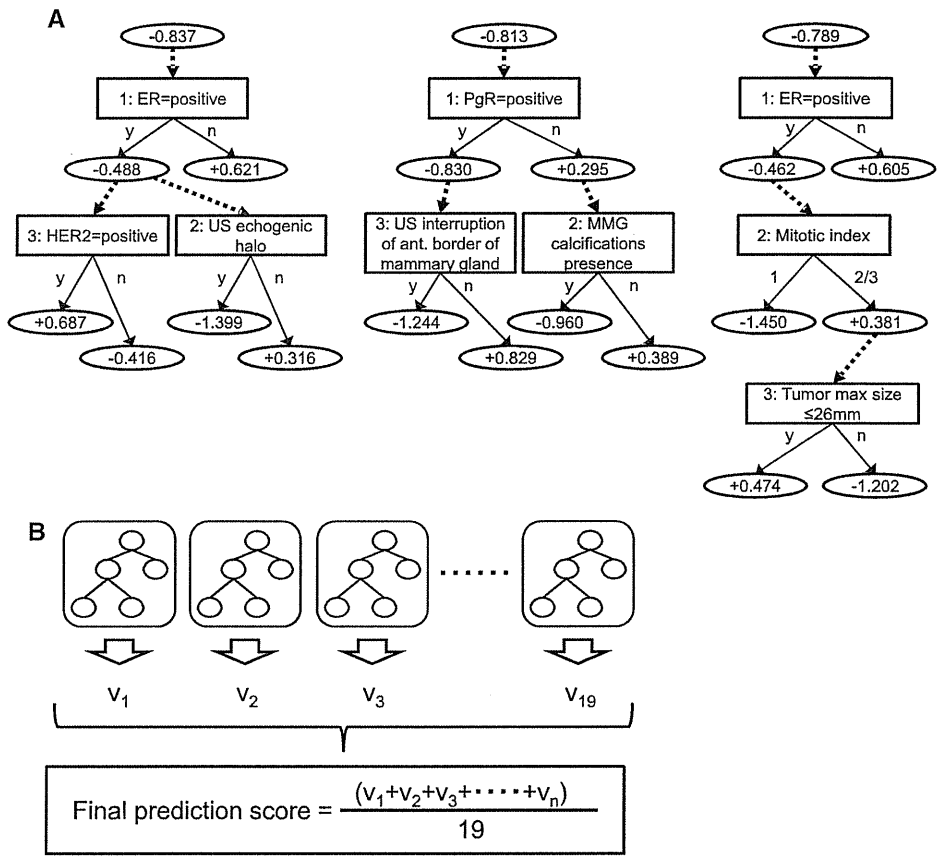

Fig. 1 ADTree-based prediction model. **a** Part of the developed ADTree models. The final prediction model consisted of 19 ADTree-based prediction models; the other 16 models are depicted in Supplementary Fig. S1. The method used to calculate the prediction score in each model is described in Supplementary Fig. S2. **b** The final prediction score was calculated by calculating the mean score of the 19 ADTree-based models. V_1, V_2 and V_{19} indicate the prediction scores of each ADTree. The probability of pCR (%) was determined using the formula $(score_{pred} - score_{min})/(score_{max} - score_{min}) \times 100$, where $score_{pred}$, $score_{min}$ and $score_{max}$ are the predicted and theoretical minimum and maximum final scores, respectively

histological/nuclear grade and interruption of the anterior border of the mammary gland on US. This MLR model yielded an AUC value of 0.754 (95 % CI 0.651–0.858, P value = 0.00019) using a subset of the validation dataset (n = 127; 46 cases were excluded because of missing values). The ADTree model outperformed the MLR model by yielding an AUC of 0.805 (95 % CI 0.716–0.894, P value < 0.0001) using the same patient dataset.

Discussion

Here, we developed a prediction model for pCR after NAC using ADTree and analyzed the model using several analyses. The validation dataset was from the OOTR N003 trial, in which patients received FEC treatment and were randomly assigned to four cycles of docetaxel alone (FEC-T) or four cycles of docetaxel plus capecitabine (FEC-TX). In the validation dataset, the AUC values for the FEC-T and FEC-TX groups were 0.789 (P value < 0.0001) and 0.788 (P value = 0.0003), respectively. The GeparQuattro study reported that adding capecitabine to preoperative docetaxel after four courses of epirubicin and cyclophosphamide did not improve the rate of pCR [22]. Accordingly, our model showed a similar performance for both the regimens.

The proportion of patients with the luminal, HER2-negative subtype was higher in the training dataset than in the validation dataset (65 and 50 %, respectively; P value = 0.008), which indicated that the training dataset included more patients with potentially chemo-insensitive cancers compared with the validation dataset. This disproportion may have led to the difference in the clinical response rate between the training and validation datasets (66 vs. 87 % after anthracycline and 85 vs. 95 % after taxane, respectively; Table 2). However, the training

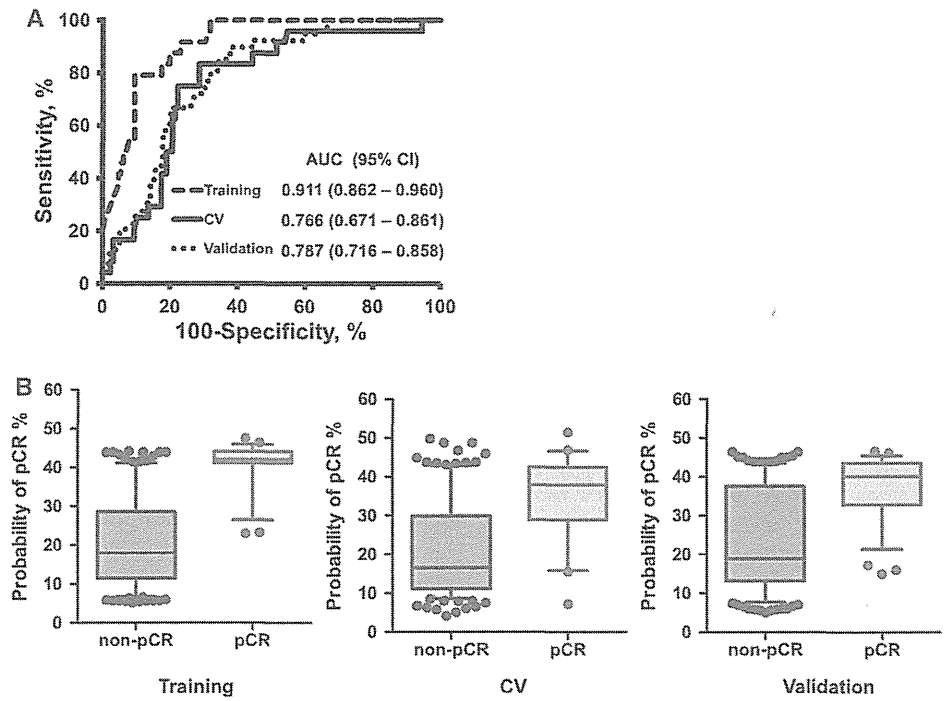

Fig. 2 **a** ROC curves and **b** the distribution of the predicted probabilities. **a** ROC curves of the prediction model. The area under the ROC curve (AUC) values were 0.766 (95 % CI 0.671–0.861, P value < 0.0001) in the CV using the training dataset, and 0.787 (95 % CI 0.716–0.858, P value < 0.0001) using the validation dataset. **b** Box plots showing the distribution of the predicted probabilities of pCR determined using our model. The *box* and *whiskers* represent the 10th, 25th, 50th, 75th and 90th percentiles, and the data below the 10th and above the 90th percentile were plotted. In each figure, the *boxes* on the *left side* of the graph represent the patients without pCR (non-pCR), and the *boxes* on the *right side* represent the patients who did achieve pCR. The model was able to discriminate pCR from non-pCR patients at significant levels (P value < 0.0001)

dataset included patients with smaller tumours compared with the validation dataset (median diameter: 26 and 29 mm, respectively; P value = 0.012; Table 1). Therefore, the differences in the clinical response rate may not be associated with differences in the pathological response rate or breast-conserving rate. Although the unequal distribution of cancer subtypes between the two datasets may affect the generalizability of our model, the result of random split analysis showed that this discrepancy hardly affected the predictive performance of our model.

There are several criteria used to define pCR after NAC. Here, we defined pCR as the absence of residual invasive cancer cells in the breast or the lymph nodes (ypT0/is + ypN0). pCR has also been defined as the complete disappearance of cancer cells from the breast and the lymph nodes (ypT0 + ypN0), and as the absence of residual cancer cells in the breast tissue, regardless of lymph node status (ypT0 + ypNX) [23]. Therefore, we evaluated the predictive performance of ADTree model using these three definitions. The AUC values were 0.728 (95 % CI 0.640–0.817; P value = 0.0002) for ypT0 + ypN0 and 0.786 (95 % CI 0.705–0.867; P value < 0.0001) for ypT0 + ypNX in the validation dataset. Although our model identified patients with and without pCR at significant levels using both the definitions, the accuracy of our model was decreased when the ypT0 + ypN0 definition was used. The rate of pCR determined by the ypT0 + ypN0 definition was lower than that for ypT0/is + ypN0 (10 vs. 16 % for the training dataset and 15 vs. 22.5 % for the validation dataset). Therefore, further evaluation using a larger dataset is needed.

It has been reported that an early clinical response to NAC might be predictive of pCR [2, 24]. As expected, the AUC values obtained using validation datasets increased to 0.820 after including the clinical responses after anthracycline treatment. Therefore, the ADTree model can provide highly accurate prediction for pCR by integrating the early clinical response to NAC.

The sensitivity analysis suggested that ER, PgR and HER2 were more important than the other variables. Among the imaging features that were generally less important than these three features, echogenic halo was the most important. Recent studies have indicated that the subgroup, mainly classified using ER and HER2 status, is strongly associated with the pathological response to NAC

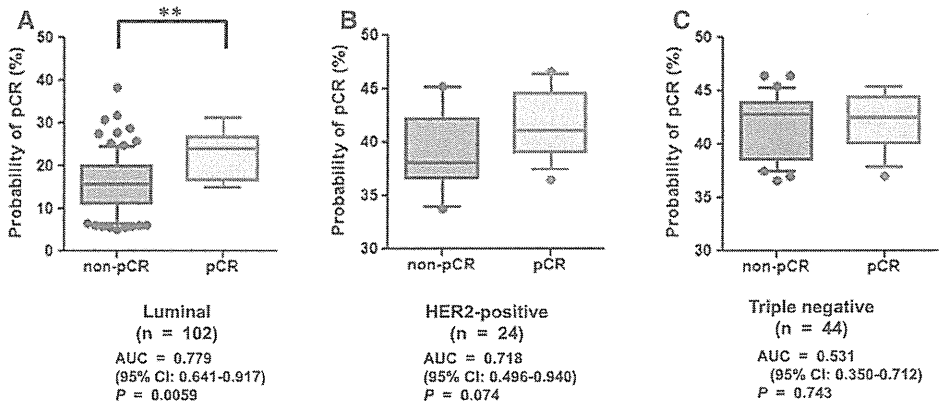

Fig. 3 Box plots showing the distribution of the predicted probabilities of pCR using the prediction model in each subgroup. In each figure, the *boxes* on the *left side* of the graph represent the patients without pCR (non-pCR), and the *boxes* on the *right side* represent the patients who did achieve pCR. Each subgroup was defined as follows: **a** luminal type (ER-positive and any HER2 status; $n = 102$); **b** HER2-positive type (ER-negative and HER2-positive; $n = 24$) and **c** triple-negative type (ER- and HER2-negative; $n = 44$)

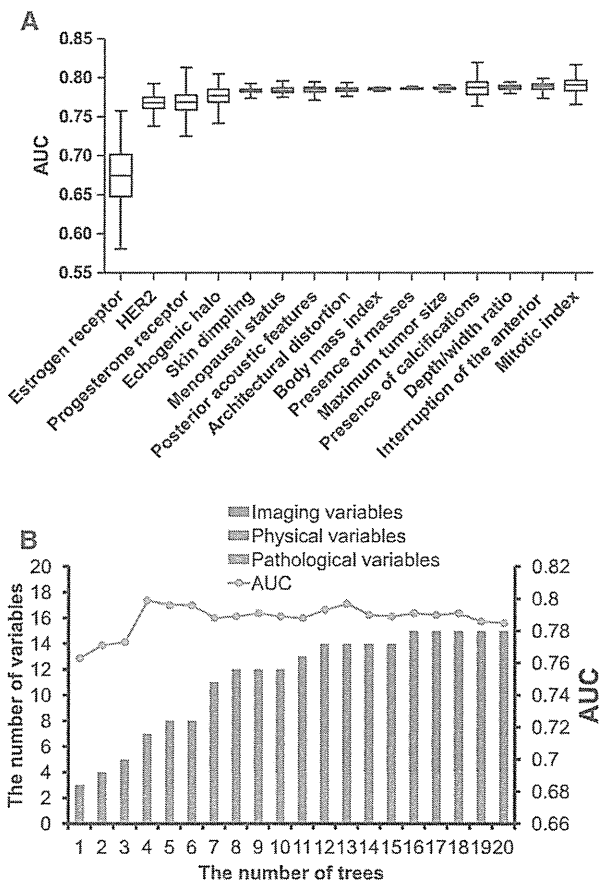

Fig. 4 **a** Sensitivity analysis and **b** pruning analysis using the validation dataset. **a** Box plots show the maximum and minimum, the 25th and 75th percentiles, and the median AUC values. **b** The horizontal axis shows the number of trees (bagging number). For each number of trees, a *bar graph* shows the number of variables and a *line graph* shows the AUC values

[8–10, 18]; however, predicting pCR within each phenotype is still challenging. The model showed relatively higher AUC values in luminal, HER2-negative and HER2-positive subtypes (Fig. 3). Meanwhile, the model did not function for the triple-negative phenotype (AUC = 0.531), which means the clinicopathological variables collected in this study show limited potential to predict chemosensitivity in this phenotype. One possible reason of this low AUC is the heterogeneity of triple-negative populations [25]. Thus, the identification of new variables, including novel genomic and epigenetic markers, and new models integrating these variables are needed to overcome this limitation.

The missing value analysis yielded an AUC value of 0.786 (95 % CI 0.785–0.787). This value was not much different from the result of bootstrap analysis (AUC = 0.791). It is particularly noteworthy that the difference between the upper and lower 95 % CI values was very small, indicating low sensitivity of the developed model to missing values. This is one of the beneficial features of the ensemble technique used in this study.

In the pruning analysis, the AUC values for the validation dataset improved according to the number of ADTrees in the prediction model (Fig. 4b). Reducing the number of trees to six eliminated many variables corresponding to imaging findings. Although the AUC value of the whole validation dataset remained high, the predictive performance for the luminal and ER-negative/HER2-positive subtypes decreased (Supplementary Fig. S4), which indicates that the variables derived from imaging findings might contribute the most to chemosensitivity prediction in both the subgroups.

Nomograms that use MLR to predict response to NAC have already been introduced [12, 13, 26]. In our study,

ADTree outperformed MLR using an identical dataset. MLR offers some advantages, particularly the use of fewer variables, which facilitates data collection and interpretation of the model. These features of each modelling method represent trade-offs that should be considered when applying the models. The combined use of multiple prediction models could enhance predictive accuracy [27]. We are currently testing the combination of our model and available nomograms in a prospective study.

There are several limitations of this study. Validation using larger databases will more accurately assess the model. The use of many features obtained from imaging studies or physical examination would reduce the number of users depending on the availability of the features. The datasets obtained from multiple institutes would contribute to strict evaluation of the model's versatility whereas such datasets sometimes introduce institute-dependent bias. In this study, we used information from individual pathology reports and the central pathology review is more preferable to evaluate the features in a single criteria. A Web-based interface to facilitate data input and prediction analysis, like the MD Anderson Cancer Centre nomogram, and an automated system to update the model will also be useful. Biomarkers of tumour response, particularly those obtained from midcourse biopsy samples, may increase the predictive accuracy. Integration with subtype-specific biomarkers is also needed to improve the accuracy of the developed model.

In conclusion, we have established a new ADTree-based method to predict pCR after NAC using variables readily collected before NAC. The model could use larger number of variables with keeping high generalization ability and showed the outperformed prediction accuracy compared with MLR as well as was tolerant to missing values and distribution bias in the datasets.

Acknowledgments We thank the doctors and data managers for data collection. We also thank the patients who participated in this study. This study was funded by research grants from the Ministry of Health, Labour and Welfare ("A study on the construction of an algorithm for multimodal therapy with biomarkers for primary breast cancer by formulation of a decision-making process", led by MT, No. H18-3JIGAN-IPPAN-007 and "Reduction and lowering of recurrence risk, toxicity and pharmacoeconomic cost by prediction of efficacy for anticancer agents in breast cancer patients", led by MT; No. H22-GANRINSHO-IPPAN-039), research funds from the Yamagata Prefectural Government and Tsuruoka City, and an International Internship Grant from the Global COE project "Centre for Frontier Medicine", Kyoto University. This study was also supported by the program "Raising Proficient Oncologists" administered by the Japanese Ministry of Education, Culture, Sports, Science and Technology.

Disclosure Dr. Hiroji Iwata has received honoraria from Chugai Pharmaceutical Co., Ltd, Japan. All remaining authors have declared no conflicts of interest.

References

1. van der Hage JH, van de Velde CCJH, Mieog SJSD (2007) Preoperative chemotherapy for women with operable breast cancer. Cochrane Database Syst Rev CD005002. doi:10.1002/14651858.CD005002.pub2
2. Bear HD, Anderson S, Smith RE, Geyer CE Jr, Mamounas EP, Fisher B, Brown AM, Robidoux A, Margolese R, Kahlenberg MS, Paik S, Soran A, Wickerham DL, Wolmark N (2006) Sequential preoperative or postoperative docetaxel added to preoperative doxorubicin plus cyclophosphamide for operable breast cancer: National Surgical Adjuvant Breast and Bowel Project Protocol B-27. J Clin Oncol 24:2019–2027. doi:10.1200/JCO.2005.04.1665
3. Fisher B, Bryant J, Wolmark N, Mamounas E, Brown A, Fisher ER, Wickerham DL, Begovic M, DeCillis A, Robidoux A, Margolese RG, Cruz AB Jr, Hoehn JL, Lees AW, Dimitrov NV, Bear HD (1998) Effect of preoperative chemotherapy on the outcome of women with operable breast cancer. J Clin Oncol 16:2672–2685
4. Mauri D, Pavlidis N, Ioannidis JP (2005) Neoadjuvant versus adjuvant systemic treatment in breast cancer: a meta-analysis. J Natl Cancer Inst 97:188–194. doi:10.1093/jnci/dji021
5. Chang J, Powles TJ, Allred DC, Ashley SE, Clark GM, Makris A, Assersohn L, Gregory RK, Osborne CK, Dowsett M (1999) Biologic markers as predictors of clinical outcome from systemic therapy for primary operable breast cancer. J Clin Oncol 17:3058–3063
6. Colleoni M, Viale G, Goldhirsch A (2009) Lessons on responsiveness to adjuvant systemic therapies learned from the neoadjuvant setting. Breast 18(Suppl 3):S137–S140. doi:10.1016/S0960-9776(09)70289-9
7. von Minckwitz G, Untch M, Nuesch E, Loibl S, Kaufmann M, Kummel S, Fasching PA, Eiermann W, Blohmer JU, Costa SD, Mehta K, Hilfrich J, Jackisch C, Gerber B, du Bois A, Huober J, Hanusch C, Konecny G, Fett W, Stickeler E, Harbeck N, Muller V, Juni P (2011) Impact of treatment characteristics on response of different breast cancer phenotypes: pooled analysis of the German neo-adjuvant chemotherapy trials. Breast Cancer Res Treat 125:145–156. doi:10.1007/s10549-010-1228-x
8. Colleoni M, Viale G, Zahrieh D, Bottiglieri L, Gelber RD, Veronesi P, Balduzzi A, Torrisi R, Luini A, Intra M, Dellapasqua S, Cardillo A, Ghisini R, Peruzzotti G, Goldhirsch A (2008) Expression of ER, PgR, HER1, HER2, and response: a study of preoperative chemotherapy. Ann Oncol 19:465–472. doi:10.1093/annonc/mdm509
9. Darb-Esfahani S, Loibl S, Muller BM, Roller M, Denkert C, Komor M, Schluns K, Blohmer JU, Budczies J, Gerber B, Noske A, du Bois A, Weichert W, Jackisch C, Dietel M, Richter K, Kaufmann M, von Minckwitz G (2009) Identification of biology-based breast cancer types with distinct predictive and prognostic features: role of steroid hormone and HER2 receptor expression in patients treated with neoadjuvant anthracycline/taxane-based chemotherapy. Breast Cancer Res 11:R69. doi:10.1186/bcr2363
10. Jones RL, Salter J, A'Hern R, Nerurkar A, Parton M, Reis-Filho JS, Smith IE, Dowsett M (2010) Relationship between oestrogen receptor status and proliferation in predicting response and long-term outcome to neoadjuvant chemotherapy for breast cancer. Breast Cancer Res Treat 119:315–323. doi:10.1007/s10549-009-0329-x
11. Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thürlimann B, Senn HJ (2011) Strategies for subtypes—dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast