Table 2
List of genes showed more than 10-fold expression ratio in V1 compared to T-47D cells.

Fold Change	Entrez gene ID	Gene symbol	Official full name
438.2	354	KLK3 (PSA)	Kallikrein-related peptidase 3
113.4	1734	DIO2	Deiodinase, iodothyronine, type II
107.3	1644	DDC	Dopa decarboxylase
68.7	4604	MYBPC1	Myosin binding protein C, slow type
57	54498	SMOX	Spermine oxidase
55.5	4837	NNMT	Nicotinamide *N*-methyltransferase
51.2	214	ALCAM	Activated leukocyte cell adhesion molecule
45.2	83539	CHST9	Carbohydrate (*N*-acetylgalactosamine 4-0) sulfotransferase 9
44	3248	HPGD	Hydroxyprostaglandin dehydrogenase 15-(NAD)
40.7	23767	FLRT3	Fibronectin leucine rich transmembrane protein 3
37.1	9073	CLDN8	Claudin 8
35.2	7704	ZBTB16	Zinc finger and BTB domain containing 16
29.8	9172	MYOM2	Myomesin (M-protein) 2
27	963	CD53	CD53 molecule
25.3	10720	UGT2B11	UDP glucoronosyltransferase 2 family, polypeptide B11
24	91464	ISX	Intestine-specific homeobox
19.5	23498	HAAO	3-Hydroxyanthranilate 3,4-dioxygenase
18.4	2668	GDNF	Glial cell derived neurotrophic factor
16.4	7364	UGT2B7	UDP glucuronosyltransferase 2 family, polypeptide B7
14.2	7365	UGT2B10	UDP glucuronosyltransferase 2 family, polypeptide B10
14	8644	AKRIC3	Aldo-keto reductase family 1, member C3
13	2554	GABRA1	Gamma-aminobutyric acid (GABA) A receptor, alpha 1
12.8	8529	CYP4F2	Cytochrome P450, family 4, subfamily F, polypeptide 2
11.6	126	ADH1C	Alcohol dehydrogenase 1C (class I), gamma polypeptide
10.7	11283	CYP4F8	Cytochrome P450, family 4, subfamily F, polypeptide 8

Gene performed real-time PCR is noted in boldface.

(Fig. 4B). Only 105 genes (27%) showed a similar level of expression in V1 and T-47D cells (Group C; ratio 0.5–2.0).

Networks of top-ranked androgen-induced genes in V1 and T-47D were determined by Ingenuity Pathway Analysis (Fig. 4C). Gene networks in V1 contained genes associated with "cell-to-cell signaling and interaction" (*CCL7, CCL11, GDNF, IL8, PDC1LG2*), "cell cycle" (*CD37, GDNF, STAT4, ZBTB16*), and "cellular development" (*GDNF, IFNA7, MPL, ZBTB16*) (left panel), while those in T-47D cells were associated with "the nervous system development and functions" (*CDH2, GLI2, NEUROD1, UNC5C*), "tissue development" (*ARHGAP26, BMX, CDH2, DLL4, GLI2, NEDD9, NEUROD1, PCDHGC3, PGF, UNC5C*), and "cell-to-cell signaling and interaction" (*ARHGAP26, BMX, CDC42EP3, CDH2, DLL4, FNPP2, GLI2, NEDD9, PGF, PTPRM, TPO, UNC5C*) (right panel). These results indicate that the expression profile of androgen-induced genes differed markedly between the variant and parental cell lines.

Of the genes classified into Group A in Fig. 4B, 25 genes showed more than a 10-fold difference in expression between V1 and T-47D cells (Table 2), with *KLK3* (PSA) exhibiting the greatest difference (438-fold). The third highest, *DDC* (L-DOPA decarboxylase; 107-fold), has been reported as an AR coactivator in prostate carcinoma [24,25], but no information is currently available regarding its involvement in breast carcinoma. DDC expression levels were validated by real-time PCR. DDC mRNA expression was 22-fold and 36-fold higher in V1 and V2 cells, respectively, than in the T-47D cells (Fig. 4D). Moreover, the DDC-inhibitor NSD-1015 decreased the DHT-induced PSA mRNA expression in variant cell lines in a dose-dependent manner (Fig. 4E). These results suggest that DDC plays an important role in increasing the AR activity in variant cell lines.

4. Discussion

This is the first report to evaluate AR activity in AI-resistant recurrent breast carcinoma. All the recurrent samples examined in this study were regarded as AI-resistant carcinoma because all the cases relapsed during the adjuvant AI therapy. In this study, PSA expression was frequently detected in recurrent breast carcinoma. The stable variant cell lines established as AI-resistance models demonstrated AR-dependent cell proliferation and overexpressed PSA. These data suggest that increased AR activity has an oncogenic role in AI-resistant breast carcinoma.

PSA expression was significantly higher in recurrent tumor tissues than in the corresponding primary lesions. PSA expression was also significantly higher in the V1 and V2 variant cell lines than in the parental T-47D cells. PSA was originally believed to be a tissue-specific protein produced by epithelial cells of the prostate gland. Several androgen-responsive elements (AREs) have been identified in the PSA promoter region [21]. PSA expression is markedly induced by DHT in AR-positive breast carcinoma cell lines, and it is now recognized as a potent androgen-induced protein in breast carcinoma cells as well as prostate carcinoma cells [26]. PSA immunolocalization has been reported in female breast carcinoma [17,27–29], and Kraus et al. [17] recently showed that PSA immunopositivity was 4% (2 of 56 cases), which is in good agreement with our data (5%). Altogether, our data and previous reports suggest that the AR activity is increased in AI-resistant recurrent lesions.

Androgenic activity is characterized by the functions of androgen-induced genes. Thus, examination of the expression profiles of androgen-induced genes in variant and T-47D cell lines was important to obtain a better understanding of androgen activity in AI-resistant cells. Interestingly, of the 390 androgen-induced genes identified by microarray analysis, only 12 (3%) were common to V1 and T-47D cells (Fig. 4A). Kabos et al. [30] reported that the patient-derived luminal breast cancer xenografts had diverse responses to endocrine therapy and had different tumor-specific ER transcriptomes. Similarly, our results showed that the V1 and T-47D cells had different AR-transcriptomes. Therefore, the molecular functions of the AR in AI-resistant recurrent lesions may be markedly different from their primary lesions. Moreover, we found that 25 androgen-induced genes whose expression was more than 10-fold higher in V1 than in T-47D cells. These genes included *DDC* (L-DOPA decarboxylase) (107-fold), known as an AR-coactivator [24], and *AKR1C5* (14-fold), which synthesizes testosterone from androstenedione [31]

(Table 2). In the present study, the growth of parental T-47D cells was slightly increased by DHT (Fig. 3C), although the proliferation effect was smaller than that mediated by estrogen (Fig. 2C). Although the T-47D cells certainly depend on ER signals for the most part, they may also have an AR-mediated proliferative pathway. In the process of adapting to estrogen-depleted and androgen- supplemented conditions, variant cell lines lost their ER and depended on the AR-mediated pathway for proliferation. In addition to increased AR expression, altered expression profiles of the androgen-induced genes may partly contribute to an increased AR activity in AI-resistant cell lines.

In our study, ER and PR LIs were significantly lower in the recurrent breast carcinoma tissues, with similar findings for variant cell lines. The PR expression is considered to indicate an intact estrogen-signaling pathway [32] and is often used as an indicator of a response to endocrine therapy for breast carcinoma [33]. A fraction of the ER-positive breast carcinomas lose the ER immunoreactivity until recurrence [34]. Of the patients who received adjuvant hormonal treatment, 9 of 49 (18%) converted from ER-positive in the primary tumor to ER-negative in the recurrent lesions [35], which is consistent with our present results.

We have established several AI-resistant cell lines that depend on an ER-mediated pathway from MCF-7 cells [36,37]. Moreover, there have been many reports that the LTED (long-term estradiol deprivation) cells overexpressed ER [5,6]. However, in this study, established cell lines from T-47D lost the ER expression. In addition, a previous study has reported that the T-47D cell line lost the ER under long-term estrogen- deprived conditions [38]. The T-47D cells may easily develop other proliferative pathways without the ER in an estrogen-free environment. These AI-resistant cell lines indicate that the character of parental cell lines determines the distinct types of resistance mechanisms.

Possible interaction between the ER and AR function has been proposed by several groups. Panet- Raymond et al. [39] reported that co-expression of the ER and AR decreased the AR trans-activation, and Takagi et al. [16] suggested that the androgen activity is suppressed in breast carcinoma by predominant estrogen activity. Therefore, these observations suggest that increased AR activity in recurrent lesions is, at least in a part, caused by decreased estrogen activity due to AI treatment.

In our study, Ki-67 LI was significantly higher in recurrent tissues, and the results of *in vitro* experiments revealed that the DHT significantly increased proliferation in variant cell lines. AR is generally considered to exert anti-proliferative effects in ER-positive breast carcinoma cells [40], but some divergent findings have been reported [41]. Recent studies suggest that the AR activity might differ with breast carcinoma subtype [14]. ER-negative breast carcinoma cell lines frequently exhibit AR- mediated cell proliferation, similar to prostate carcinoma; cross-talk between the AR and HER2 signaling pathway has been suggested [12,13]. Harvell et al. [42] demonstrated that the AR level in breast carcinoma after neoadjuvant AI therapy increased in AI non-responders, but decreased in responders, suggesting that the AR may play an important role in resistance to endocrine therapy in ER-positive breast carcinoma. Foekens et al. [43] reported that the PSA level in breast tumor cytosol correlated with poor response to tamoxifen therapy in the recurrent disease. Altogether with these reports, our results indicate that the AR activity may be more oncogenic in the AI-resistant breast carcinoma.

PSA expression was not always observed in the recurrent tissues in our study, suggesting that the induction of an AR-mediated proliferation pathway is one of the AI-resistance mechanisms.

Kabos et al. [30] suggested that the acquired resistance mechanisms differ in each individual tumor, which may explain our findings. We are currently establishing several breast carcinoma cell lines to use as AI-resistance models under estrogen-depleted and androgen-supplemented conditions. Androgen metabolite-dependent and estrogen-depletion-resistant cells derived from the MCF-7 cells were recently reported to show dose-dependent activation of ER functions by the estrogenic androgen 5α-androstane-3β,17β-diol (3β-diol) and markedly decreased AR expression [36]. In addition, several ER-independent proliferative pathways have been reported as AI-resistance mechanisms, including up-regulation of the ER-mediated pathway [5,6], the growth factor receptor- mediated pathways [7,8], mitogen-activated protein kinase (MAPK), and phosphatidylinositide 3- kinase (PI3K)/Akt [6,9,37]. Therefore, acquired AI-resistance mechanisms are suggested to be diverse and appropriately targeted therapies, according to case are required.

The AR was recently reported to have therapeutic potential in some breast carcinomas. A phase II trial of bicalutamide for ER-negative/AR-positive metastatic breast carcinoma showed the efficacy of AR-blockade in these patients [44], and a phase II trial evaluating the efficacy of the AR-inhibitor enzalutamide in combination with exemestane in advanced ER-positive breast cancer patients is ongoing (NCT02007512). The results of our present study suggest that AR inhibitors may be effective in a select group of AI-resistant breast carcinoma patients, and the PSA status may be a useful indicator of the response. Further investigations are needed to clarify the clinical significance of AR inhibitors in AI-resistant breast carcinoma.

In summary, examination of the immunohistochemical features of 21 AI-resistant recurrent breast carcinomas demonstrated that the PSA expression and the Ki-67 LI were increased and ER LI and PR LI were decreased in the recurrent lesions compared to the corresponding primary lesions. Moreover, we established two AI-resistant breast carcinoma cell lines and demonstrated that both PSA expression and AR-mediated cell proliferation were increased in these cell lines compared to a parental cell line. The expression profiles of androgen-induced genes in AI-resistant cells differed markedly from parental cells. These results suggest that the increased oncogenic AR activity in recurrent breast carcinoma is a mechanism of acquired AI-resistance, and the AR inhibitors may be effective in a select group of patients.

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References

[1] J.F. Forbes, J. Cuzick, A. Buzdar, A. Howell, J.S. Tobias, M. Baum, et al., Effect of anastrozole and tamoxifen as adjuvant treatment for early- stage breast cancer: 100-month analysis of the ATAC trial, Lancet Oncol. 9 (2008) 45–53.
[2] H. Mouridsen, A. Giobbie-Hurder, A. Goldhirsch, B. Thürlimann, R. Paridaens, I. Smith, et al., Letrozole therapy alone or in sequence with tamoxifen in women with breast cancer, New Engl. J. Med. 361 (2009) 766–776.

[3] R.C. Coombes, L.S. Kilburn, C.F. Snowdon, R. Paridaens, R.E. Coleman, S.E. Jones, et al., Survival and safety of exemestane versus tamoxifen after 2–3 years' tamoxifen treatment (Intergroup Exemestane Study): a randomised controlled trial, Lancet 369 (2007) 559–570.
[4] S.R. Johnston, L.A. Martin, J. Head, I. Smith, M. Dowsett, Aromatase inhibitors: combinations with fulvestrant or signal transduction inhibitors as a strategy to overcome endocrine resistance, J. Steroid Biochem. Mol. Biol. 95 (2005) 173–181.
[5] S. Masamura, S.J. Santner, D.F. Heitjan, R.J. Santen, Estrogen deprivation causes estradiol hypersensitivity in human breast cancer cells, J. Clin. Endocrinol. Metab. 80 (1995) 2918–2925.
[6] R. Santen, M.H. Jeng, J.P. Wang, R. Song, S. Masamura, R. McPherson, et al., Adaptive hypersensitivity to estradiol: potential mechanism for secondary hormonal responses in breast cancer patients, J. Steroid Biochem. Mol. Biol. 79 (2001) 115–125.
[7] G. Bunone, P.A. Briand, R.J. Miksicek, D. Picard, Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation, EMBO J. 15 (1996) 2174–2183.
[8] D.L. Miller, D. el-Ashry, A.L. Cheville, Y. Liu, S.W. McLeskey, F.G. Kern, Emergence of MCF-7 cells overexpressing a transfected epidermal growth factor receptor (EGFR) under estrogen- depleted conditions: evidence for a role of EGFR in breast cancer growth and progression, Cell Growth Differ. 5 (1994) 1263–1274.
[9] R.A. Campbell, P. Bhat- Nakshatri, N.M. Patel, D. Constantinidou, S. Ali, H. Nakshatri, Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance, J. Biol. Chem. 276 (2001) 9817–9824.
[10] T. Suzuki, Y. Miki, Y. Nakamura, T. Moriya, K. Ito, N. Ohuchi, et al., Sex steroid-producing enzymes in human breast cancer, Endocr. Relat. Cancer 12 (2005) 701–720.
[11] F.E. Vera-Badillo, A.J. Templeton, P. de Gouveia, I. Diaz-Padilla, P.L. Bedard, M. Al-Mubarak, et al., Androgen receptor expression and outcomes in early breast cancer: a systematic review and meta-analysis, J. Natl. Cancer Inst. 106 (2014) djt319.
[12] J.L. Robinson, S. Macarthur, C.S. Ross-Innes, W.D. Tilley, D.E. Neal, I.G. Mills, et al., Androgen receptor driven transcription in molecular apocrine breast cancer is mediated by FoxA1, EMBO J. 30 (2011) 3019–3027.
[13] M. Ni, Y. Chen, E. Lim, H. Wimberly, S.T. Bailey, Y. Imai, et al., Targeting androgen receptor in estrogen receptor-negative breast cancer, Cancer Cell 20 (2011) 119–131.
[14] T.E. Hickey, J.L. Robinson, J.S. Carroll, Tilley W.D. Minireview, The androgen receptor in breast tissues: growth inhibitor, tumor suppressor, oncogene, Mol. Endocrinol. 26 (2012) 1252–1267.
[15] T. Suzuki, Y. Miki, T. Moriya, J. Akahira, T. Ishida, H. Hirakawa, et al., 5Alpha-reductase type 1 and aromatase in breast carcinoma as regulators of in situ androgen production, Int. J. Cancer 120 (2007) 285–291.
[16] K. Takagi, Y. Miki, S. Nagasaki, H. Hirakawa, Y. Onodera, J. Akahira, et al., Increased intratumoral androgens in human breast carcinoma following aromatase inhibitor exemestane treatment, Endocr. Relat. Cancer 17 (2010) 415–430.
[17] T.S. Kraus, C. Cohen, M.T. Siddiqui, Prostate-specific antigen and hormone receptor expression in male and female breast carcinoma, Diagn. Pathol. 5 (2010) 63.
[18] Y. Yamaguchi, H. Takei, K. Suemasu, Y. Kobayashi, M. Kurosumi, N. Harada, et al., Tumor-stromal interaction through the estrogen-signaling pathway in human breast cancer, Cancer Res. 65 (2005) 4653–4662.
[19] Y. Omoto, Y. Kobayashi, K. Nishida, E. Tsuchiya, H. Eguchi, K. Nakagawa, et al., Expression, function, and clinical implications of the estrogen receptor beta in human lung cancers, Biochem. Biophys. Res. Commun. 285 (2001) 340–347.
[20] A. Mizokami, A. Gotoh, H. Yamada, E.T. Keller, T. Matsumoto, Tumor necrosis factor-alpha represses androgen sensitivity in the LNCaP prostate cancer cell line, J Urol. 164 (2000) 800–805.
[21] P.H. Riegman, R.J. Vlietstra, J.A. van der Korput, A.O. Brinkmann, J. Trapman, The promoter of the prostate-specific antigen gene contains a functional androgen responsive element, Mol. Endocrinol. 5 (1991) 1921–1930.
[22] A. Migliaccio, G. Castoria, M. Di Domenico, A. de Falco, A. Bilancio, M. Lombardi, et al., Steroid-induced androgen receptor-oestradiol receptor beta-Src complex triggers prostate cancer cell proliferation, EMBO J. 19 (2000) 5406–5417.
[23] P. Kastner, A. Krust, B. Turcotte, U. Stropp, L. Tora, H. Gronemeyer, et al., Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B, EMBO J. 9 (1990) 1603–1614.
[24] L.A. Wafa, H. Cheng, M.A. Rao, C.C. Nelson, M. Cox, M. Hirst, et al., Isolation and identification of L-dopa decarboxylase as a protein that binds to and enhances transcriptional activity of the androgen receptor using the repressed transactivator yeast two-hybrid system, Biochem. J. 375 (2003) 373–383.
[25] G. Koutalellis, K. Stravodimos, M. Avgeris, K. Mavridis, A. Scorilas, A. Lazaris, et al., L-dopa decarboxylase (DDC) gene expression is related to outcome in patients with prostate cancer, BJU Int. 110 (2012) E267–E273.
[26] A. Magklara, L. Grass, E.P. Diamandis, Differential steroid hormone regulation of human glandular kallikrein (hK2) and prostate-specific antigen (PSA) in breast cancer cell lines, Breast Cancer Res. Treat. 59 (2000) 263–270.
[27] A. Tanaka, T. Kamiakito, N. Takayashiki, S. Sakurai, K. Saito, Fibroblast growth factor 8 expression in breast carcinoma: associations with androgen receptor and prostate-specific antigen expressions, Virchows Arch. 441 (2002) 380–384.
[28] S. Ilvan, V. Celik, I. Cetinaslan, Z. Calay, M. Ferahman, Immunohistochemical analysis of prostate-specific antigen in female breast cancer, J. BUON 9 (2004) 183–186.
[29] D. Narita, M. Raica, C. Suciu, A. Cîmpean, A. Anghel, Immunohistochemical expression of androgen receptor and prostate-specific antigen in breast cancer, Folia Histochem. Cytobiol. 44 (2006) 165–172.
[30] P. Kabos, J. Finlay- Schultz, C. Li, E. Kline, C. Finlayson, J. Wisell, et al., Patient-derived luminal breast cancer xenografts retain hormone receptor heterogeneity and help define unique estrogen-dependent gene signatures, Breast Cancer Res. Treat. 135 (2012) 415–432.
[31] F. Labrie, V. Luu-The, S.X. Lin, C. Labrie, J. Simard, R. Breton, et al., The key role of 17 beta-hydroxysteroid dehydrogenases in sex steroid biology, Steroids 62 (1997) 148–158.
[32] M. Dowsett, S.R. Ebbs, J.M. Dixon, A. Skene, C. Griffith, I. Boeddinghaus, et al., Biomarker changes during neoadjuvant anastrozole, tamoxifen, or the combination: influence of hormonal status and HER-2 in breast cancer – a study from the IMPACT trialists, J. Clin. Oncol. 23 (2005) 2477–2492.
[33] V.J. Bardou, G. Arpino, R.M. Elledge, C.K. Osborne, G.M. Clark, Progesterone receptor status significantly improves outcome prediction over estrogen receptor status alone for adjuvant endocrine therapy in two large breast cancer databases, J. Clin. Oncol. 21 (2003) 1973–1979.
[34] G. Bogina, L. Bortesi, M. Marconi, M. Venturini, G. Lunardi, F. Coati, et al., Comparison of hormonal receptor and HER-2 status between breast primary tumours and relapsing tumours: clinical implications of progesterone receptor loss, Virchows Arch. 459 (2011) 1–10.
[35] P.K. Idirisinghe, A.A. Thike, P.Y. Cheok, G.M. Tse, P.C. Lui, S. Fook-Chong, et al., Hormone receptor and c-ERBB2 status in distant metastatic and locally recurrent breast cancer. Pathologic correlations and clinical significance, Am. J. Clin. Pathol. 133 (2010) 416–429.
[36] T. Hanamura, T. Niwa, S. Nishikawa, H. Konno, T. Gohno, C. Tazawa, et al., Androgen metabolite-dependent growth of hormone receptor-positive breast cancer as a possible aromatase inhibitor-resistance mechanism, Breast Cancer Res. Treat. 139 (2013) 731–740.
[37] N. Fujiki, H. Konno, Y. Kaneko, T. Gohno, T. Hanamura, K. Imami, et al., Estrogen response element-GFP (ERE-GFP) introduced MCF-7 cells demonstrated the coexistence of multiple estrogen-deprivation resistant mechanisms, J. Steroid Biochem. Mol. Biol. (2013) .
[38] C.S. Murphy, J.J. Pink, V.C. Jordan, Characterization of a receptor-negative, hormone-nonresponsive clone derived from a T47D human breast cancer cell line kept under estrogen-free conditions, Cancer Res. 50 (1990) 7285–7292.
[39] V. Panet-Raymond, B. Gottlieb, L.K. Beitel, L. Pinsky, M.A. Trifiro, Interactions between androgen and estrogen receptors and the effects on their transactivational properties, Mol. Cell. Endocrinol. 167 (2000) 139–150.
[40] A.A. Peters, G. Buchanan, C. Ricciardelli, T. Bianco-Miotto, M.M. Centenera, J.M. Harris, et al., Androgen receptor inhibits estrogen receptor-alpha activity and is prognostic in breast cancer, Cancer Res. 69 (2009) 6131–6140.
[41] S.N. Birrell, J.M. Bentel, T.E. Hickey, C. Ricciardelli, M.A. Weger, D.J. Horsfall, et al., Androgens induce divergent proliferative responses in human breast cancer cell lines, J. Steroid Biochem. Mol. Biol. 52 (1995) 459–467.
[42] D.M. Harvell, J.K. Richer, M. Singh, N. Spoelstra, C. Finlayson, V.F. Borges, et al., Estrogen regulated gene expression in response to neoadjuvant endocrine therapy of breast cancers: tamoxifen agonist effects dominate in the presence of an aromatase inhibitor, Breast Cancer Res. Treat. 112 (2008) 489–501.
[43] J.A. Foekens, E.P. Diamandis, H. Yu, M.P. Look, M.E. Meijer-van Gelder, W.L. van Putten, et al., Expression of prostate-specific antigen (PSA) correlates with poor response to tamoxifen therapy in recurrent breast cancer, Br. J. Cancer 79 (1999) 888–894.
[44] A. Gucalp, S. Tolaney, S.J. Isakoff, J.N. Ingle, M.C. Liu, L.A. Carey, I.I. et al. Phase, Trial of bicalutamide in patients with androgen receptor-positive, estrogen receptor-negative metastatic breast cancer, Clin. Cancer Res. 19 (2013) 5505–5512.
[45] A. Goldhirsch, W.C. Wood, A.S. Coates, R.D. Gelber, B. Thürlimann, H.J. Senn, et al., 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 Cancer 2011, Ann. Oncol. 22 (2011) 1736–1747.

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Intratumoral androgen metabolism and actions in invasive lobular carcinoma of the breast

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Invasive lobular carcinoma (ILC) accounts for approximately 10% of all breast carcinomas and is characterized by higher levels of androgen receptor (AR) compared to invasive ductal carcinoma (IDC). Despite this potentially androgen-responsive environment, the combined importance of AR and androgen metabolism in non-neoplastic lobules and lobular carcinoma remains unknown. Therefore, in this study, we evaluated the status of pivotal androgen-producing enzymes 17β-hydroxysteroid dehydrogenase type 5 (17βHSD5) and 5α-reductase type 1 (5αRed1) in 178 cases of ILC and surrounding histologically non-neoplastic lobular tissue using immunohistochemistry. Androgen receptor prevalence was higher but androgenic enzymes lower in ILC than non-neoplastic lobules. In ILC cases the status of 5αRed1 and 17βHSD5 was inversely correlated with tumor size ($P = 0.0053$) and nuclear grade ($P = 0.0290$), and significantly associated with better overall survival of the patients ($P = 0.0059$). Based on these findings, we hypothesized that androgen signaling could act as a tumor suppressor. As previous studies suggested that androgens might partially act by increasing levels of the estrogen inactivating enzyme 17β-hydroxysteroid dehydrogenase type 2 (17βHSD2) in IDC tissues, this was reasonably considered a potential mechanism of androgen actions. Significantly positive correlation was detected between the status of androgenic enzymes and 17βHSD2 ($P < 0.0001$) and intratumoral 17βHSD2 was inversely correlated with tumor size in ILC ($P = 0.0075$). These correlations suggest one protective mode of androgen action could be through modulation of estrogen metabolism. Results of our present study indicated that androgen-producing enzymes could play pivotal protective roles in AR-enriched ILC cases.

Invasive lobular carcinoma accounts for approximately 10% of all breast invasive carcinoma cases,[1,2] and its incidence is increasing among postmenopausal women.[3,4] Invasive lobular carcinoma is distinguished from IDC by pathological, biological, and molecular characteristics. Invasive lobular carcinoma is especially characterized by the loss of E-cadherin in carcinoma cells,[5–7] which plays a hallmark role in histopathological differentiation from IDC. In addition, ILC patients generally have larger tumor size and more abundant ER/PR content but, paradoxically, lower cell proliferation compared to IDC patients.[1,8,9] This very abundance of ER/PR has been postulated to account for a better response to adjuvant hormone therapy in ILC compared to IDC patients.[8]

In addition to estrogens and progesterones, androgens have been recently considered to play pivotal roles in both the presence and absence of ER and PR. Androgens exert their actions through AR, the presence of which is reported in the great majority of breast cancer patients, more frequently than ER and PR.[10,11] The presence of AR is particularly marked in ILC with expression in 90% of ILC patients compared to 56% in IDC patients.[8,12] In addition, the clinical or biological significance of androgen signaling is different depending on the presence or absence of ER and the equilibrium of signaling between these male and female hormones is proposed to exert an important impact upon the biological or clinical significance of the patients.[13–15] In general, androgen actions in the presence of ER antagonize ER binding, subsequently resulting in suppression of estrogen-mediated tumorigenesis. This hypothesis has been supported by results of *in vitro* studies indicating suppressed estrogen signaling by direct binding of AR to ER responsive element[15] and suppression of cell proliferation by androgen treatment.[16–18]

The delicate equilibrium between androgen and estrogen signaling in breast carcinoma cells is first and foremost regulated by the levels of androgen and estrogen receptors[15,19,20] but an additional level of regulation comes from intracrine metabolism of steroid hormones.[21] It is entirely true that the status of intratumoral androgen metabolism has been less frequently explored than that of estrogen, but the contribution of this intracrine system to the generation of androgen signaling within tissues is supported by the significantly increased localized levels of potent androgen DHT in postmenopausal breast cancers.[22,23] Therefore, it is also pivotal to study the status of intratumoral androgen metabolism in breast cancer. The two principle androgen metabolizing enzymes are 17βHSD5 and 5αRed1.[24] We have previously reported that the co-expression of AR and 5αRed1 in invasive ductal carcinomas was indeed associated with better clinical outcome of the patients with IDC,[25] which is consistent with the overall roles of androgens as tumor suppressors in ER-positive or luminal-type breast carcinoma.

In addition, AIs, considered the gold standard of endocrine therapy of ER-positive postmenopausal breast cancer patients, have been reported to exert antitumor effects through not only decreasing the levels of estrogens available for carcinoma cells but also increasing intratumoral androgen concentrations, most probably due to the precursor–product relationship between intratumoral androgens and estrogens.[26–28] Recently we showed that the increased androgen concentration in breast cancer tissues following AI exemestane treatment was significantly associated with an increment of 17βHSD2. The latter is well known to decrease the levels of potent estrogens, estradiol, and thus overall estrogen signaling.[26,27] In addition, we also reported an increased expression level of 17βHSD2 by DHT or exemestane treatment in a breast carcinoma cell line, suggesting 17βHSD2 expression does reflect intratumoral androgenic actions in breast cancer tissues and could possibly account for the tumor suppressive actions of androgen signaling in estrogen-dependent breast cancers.

Invasive lobular carcinoma has been reported to have more abundant AR in carcinoma cells than IDC, as described above,[8,12] but the clinical and biological significance of androgen signaling has remained largely unexplored. In studies attempting this comparison, the interpretation of their findings has been extremely difficult due to the relative rarity of ILC and the high prevalence of AR in ILC patients. In addition, the prevalence of AR and androgen-synthesizing enzymes in non-neoplastic human lobular tissues as compared to carcinomas has been virtually unknown. Therefore, in this study, we examined the intratumoral status of AR and androgen-producing enzymes in non-neoplastic lobules and ILC tissues in order to assess the associations between increased androgen production and/or signaling and various clinicopathological factors of ILC cases, including clinical outcome of the patients. We also studied the status of the androgen-induced estrogen metabolizing enzyme 17βHSD2 and compared its status with that of the androgenic enzymes to further explore the mechanisms of androgen actions in ILC patients.

Materials and Methods

Formalin-fixed, paraffin-embedded tissues. Invasive lobular carcinoma cases examined in this study were all surgical specimens retrieved from surgical pathology files of Tohoku University Hospital (Sendai, Japan), Sagara Hospital (Kagoshima, Japan), and Tohoku Kosai hospital (Sendai, Japan). None of the patients received hormonal therapy prior to surgery; in the few patients that had received chemotherapy before surgery, results were no different from those who did not. The mean age of the patients was 57 years (range, 32–91). Clinicopathological findings including menopausal status, stage, nuclear grade, ER, PR, Her2, and Ki-67 were available in all of the cohorts examined, and data regarding the tumor size and clinical outcome available in the cohorts of Tohoku University Hospital and Kosai Hospital. Invasive ductal carcinoma cases examined in this study were also retrieved from surgical pathology files of Tohoku University Hospital. The mean age was 59 years (range, 34–82). All the specimens had been fixed with 10% formalin and embedded in paraffin. The research protocol was approved by the Ethics Committee at Tohoku University School of Medicine and review boards of all participating institutions.

Fresh frozen tissues. In addition to the 10% formalin-fixed and paraffin-embedded tissues described above, four ILC and four IDC fresh-frozen specimens were available for RT-PCR study. These specimens were obtained from Sagara Hospital and Tohoku University Hospital in 2010 and 2011. The clinicopathological findings, including age and the ER/PR and Her2 status of the patients, are summarized in Table 1. The research protocol was approved by the Ethics Committee of Tohoku University School of Medicine and the Sagara Hospital review board.

Immunohistochemistry. Staining for 17βHSD5, 5αRed1, AR, and 17βHSD2 was carried out, as summarized in Table 2. A Histofine Kit (Nichirei Bioscience, Tokyo, Japan), based on the biotin–streptavidin method, was used for immunohistochemical staining in this study. After deparaffinization, antigen retrieval was carried out by heating the slides in an autoclave at 120°C for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate [pH 6.0]) for immunostaining of 17βHSD5 and AR. The antigen–antibody complex was visualized with 3, 3′-diaminobenzidine solution (1 mM 3, 3′-diaminobenzidine, 50 mM Tris–HCl buffer [pH 7.6], and 0.006% H_2O_2) and counter-stained with hematoxylin.

Evaluation of immunoreactivity. Androgen receptor immunoreactivity was assessed by LI.[29] Immunoreactivity was detected in the nuclei. We counted more than 1000 breast carcinoma cells in each case. Subsequently the percentage of immunoreactivity was determined. Labeling index was used to obtain a proportion of immunoreactive tumor cells. The cases with <10% positivity were considered negative according to our previous reports.[25] Both 17βHSD5 and 5αRed1 immunoreactivity was detected in the cytoplasm, and the cases were tentatively classified into the following two groups: negative, 0–50%; positive, 50–100%, as previously described.[28] Immunoreactivity of 17βHSD2 was also detected in the cytoplasm and classified into two groups: negative, 0–10%; and positive, 10–100%.[30]

Reverse transcription–polymerase chain reaction. Total RNA of IDC and ILC tissues were extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). Complementary DNA for RT^2 qPCR was synthesized using RT^2 First Strand Kit in accordance with manufacturer's protocol. All IDC and ILC cases

Table 1. Characteristics of invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) cases used for RT-PCR analysis

	Age, years	ER†	PR†	Her2
IDC-1	47	5	5	–
IDC-2	76	5	3	–
IDC-3	60	5	4	–
IDC-4	46	5	4	–
ILC-1	60	5	5	–
ILC-2	55	5	5	–
ILC-3	52	5	4	–
ILC-4	48	5	5	–

†Positivity is presented as: 5, 90–100%; 4, 50–90%; 3, 10–50%; 2, 1–10%; 1, 0–1%; 0, 0%. –, Not detected; ER, Eestrogen receptor; PR, Pprogesterone receptor; Her2, human epidermal growth factor receptor 2.

Table 2. Antibodies used for immunostaining of androgen-producing enzymes in 178 cases of invasive lobular carcinoma and surrounding non-neoplastic lobular tissue

Primary antibody	Dilution	Source	Host	Antigen retrieval
17βHSD5	1:200	Sigma (St. Louis, MO, USA)	Mouse	Autoclave
5αRed1	1:1000	Abcam (Cambridge, UK)	Goat	None
AR	1:50	Dako (Kyoto, Japan)	Mouse	Autoclave
17βHSD2	1:200	Proteintech (Chicago, IL, USA)	Rabbit	None

17βHSD2, 17β-hydroxysteroid dehydrogenase type 2; 17βHSD5, 17β-hydroxysteroid dehydrogenase type5; 5α-Red1, 5α-Reductase type 1; AR, Aandrogen receptor.

were analyzed for 17βHSD2 expression using RT^2 SYBR Green qPCR Mastermixes (Qiagen, Hilden, Germany). Polymerase chain reaction was carried out in an ABI7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Data analyses were carried out with the web-based software package for the RT^2 Profiler PCR Array Data Analysis (http://www.sabiosciences.com/pcr/arrayanalysis.php).

Statistical analysis. All statistical analyses were carried out using JMP Pro 9.0.2 (SAS Institute Japan, Tokyo, Japan). The associations between androgenic enzymes and clinicopathological parameters of the cases examined were evaluated using Student's *t*-test or the χ^2-test depending on whether the variable was continuous or categorical. Both DFS and OS were analyzed according to the Kaplan–Meier method, and the statistical significance was assessed using the log–rank test. Univariate and multivariate analyses were employed in this study using Cox's proportional hazard model. These analyses were limited to the cases with at least 5 years follow-up duration (2008 or earlier surgical date) due to the long latency period associated with this neoplasm.

Results

Immunoreactivity in ILC cases and adjacent non-neoplastic lobules. Androgen receptor immunoreactivity was detected in the nuclei of tumor cells, and 17βHSD5 and 5αRed1 immunoreactivity in the cytoplasm of tumor cells (Fig. 1). Androgen receptor was highly prevalent with positive nuclei detected in almost all the carcinoma cells in ILC. In ILC, AR-positive cases (defined as >10% LI) were 97.8% (174 cases out of 178) and the mean value of AR LI was 90.6 ± 1.4 (range, 0–100%). The proportion of 17βHSD5- and 5αRed1-positive cases (defined as >50% tumor cells of cytoplasmic immunoreactivity) were 61.8% and 53.4%, respectively, with 34.8% double-positive for both enzymes.

The status of AR and androgenic enzymes in non-neoplastic lobular epithelial cells adjacent to ILC was also examined in this study. Androgen receptor immunoreactivity was also detected in non-neoplastic lobular epithelium but its prevalence was lower than that in carcinoma. When quantified, all non-neoplastic lobular epithelium in the cases examined (n = 28) were AR-positive (defined as >10% LI) and the mean value of AR LI was 63.2 ± 4.0 (range, 30–95%). The 17βHSD5- and 5αRed1-positive cases (defined as >50% tumor cells of cytoplasmic immunoreactivity) constituted 89.3% (25 cases out of 28) and 71.4% (20 out of 28), respectively. Immunoreactivity of both AR and androgenic enzymes were only sporadically detected in basal or myoepithelial cells of the non-neoplastic lobules adjacent to ILC.

The AR LI score was significantly higher in ILC than adjacent non-neoplastic lobular epithelium ($P < 0.0001$) but 17βHSD5 and 5αRed1 were significantly higher in non-neoplastic adjacent lobular epithelium than in ILC ($P = 0.0028$ and $P = 0.0128$, respectively). The status of AR in non-neoplastic lobular epithelium was similar to that in ductal epithelium, although not quantified.

Correlations of intratumoral androgen metabolizing enzymes and clinicopathological factors in ILC cases. The AR-negative cases (n = 4) were excluded to analyze the effects of androgen metabolizing enzymes on AR-mediated actions in ILC. We tentatively classified the 174 AR-positive ILC cases into the following two groups: a 17βHSD5 and 5αRed1 double-negative cohort of patients lacking any form of androgen synthesis (45 samples); and a group encompassing all other cases (129 samples) according to the potential local production of androgens. The associations between the status of intratumoral androgenic enzymes and clinicopathological parameters of the patients are summarized in Table 3. Age, menopausal status, ER, Her2, and Ki-67 status were not correlated with the status of intratumoral androgenic enzymes. Stage and the status of PR tended to be inversely correlated with that of intratumoral androgenic enzymes ($P = 0.0720$ and $P = 0.0663$, respectively). Tumor size and nuclear grade were inversely correlated with the status of intratumoral androgen metabolizing enzymes ($P = 0.0053$ and $P = 0.0290$, respectively). The mean tumor size was also higher in the 17βHSD5–5αRed1 double-negative group of patients (Fig. 2), suggesting the correlation of tumor size with the presence of intratumoral androgenic enzymes.

Correlation between status of intratumoral androgenic enzymes and clinical outcome. Data regarding OS and DFS were available for cases from Tohoku University Hospital and Tohoku Kosai Hospital. As the cases were limited to those that had a follow-up period >5 years, the number available for this analysis was 72. Despite the relatively small number of cases examined, the absence of intratumoral androgenic enzymes was significantly associated with adverse OS (Fig. 3a) but not with DFS (Fig. 3b) and this association still remained significant in the multivariate analysis (model including ER, PR, stage, and enzyme status; data not shown).

Androgen and 17βHSD2-producing enzymes in ILC. It was previously reported that 17βHSD2 reflected intratumoral androgenic actions in breast cancer.[27] In this study, 17βHSD2 mRNA levels were quantified in the four ILC and four IDC cases summarized in Table 1 with RT^2 qPCR, in order to compare 17βHSD2 mRNA expression between ILC and IDC cases. As illustrated in Figure 4(a), the amount of 17βHSD2 mRNA expression was 4.8-fold higher in ILC than IDC cases ($P = 0.0775$). The 17βHSD2 immunoreactivity was also examined in order to further explore the significance of this particular enzyme with relation to androgen actions. Fifty-four IDC cases (Tohoku University Hospital) were selected so that ER, PR, Her2, Ki-67, stage, AR, 17βHSD5, and 5αRed1 status did not differ significantly with 46 ILC cases (Tohoku University Hospital). Immunoreactivity of 17βHSD2 was detected in the

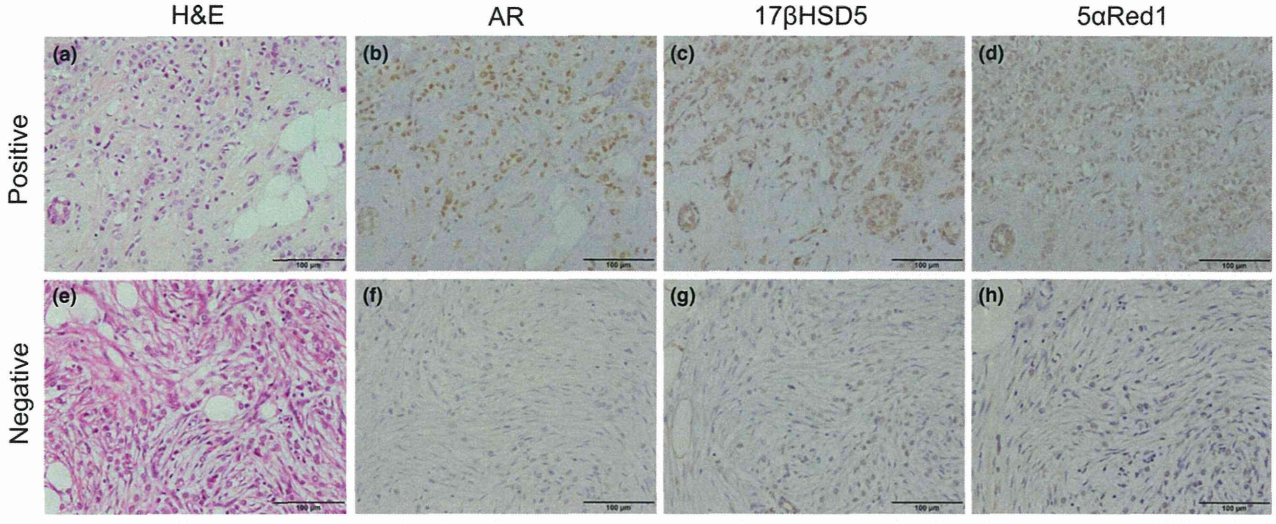

Fig. 1. Representative illustrations of androgen receptor (AR), 17β-hydroxysteroid dehydrogenase type 5 (17βHSD5), and 5α-Reductase type 1 (5αRed1) immunohistochemistry in AR+/17βHSD5+/5αRed1+ (b–d) and AR−/17βHSD5−/5αRed1− (f–h) invasive lobular carcinoma cases. Hematoxylin–eosin staining (a,e). Androgen receptor was immunolocalized in the nuclei of carcinoma cells (b,f), and 17βHSD5 (c,g) and 5αRed1 (d,h) immunolocalized in the cytoplasm of carcinoma cells. Scale bar = 100 μm.

Table 3. Associations between the status of intratumoral androgenic enzymes and clinicopathological parameters in androgen receptor (AR)-positive invasive lobular carcinoma (ILC) cases (n = 174)

	17βHSD5/5αRed1		P-value
	−/− n = 45 (25.9%)	Others n = 129 (74.1%)	
Age,† (years)	56.0 ± 1.9	57.9 ± 1.0	0.3632
Menopausal status, n (%)			
Premenopausal	22 (12.7%)	46 (26.4%)	0.1173
Postmenopausal	23 (13.2%)	83 (47.7%)	
Stage, n (%)			
1	14 (8.0%)	60 (34.5%)	0.0720
2 + 3	31 (17.8%)	69 (39.7%)	
Tumor size,‡ n (%)			
<20 mm	12 (9.8%)	53 (43.1%)	0.0053
≥20 mm	24 (19.5%)	34 (27.6%)	
Nuclear grade, n (%)			
1	7 (4.0%)	42 (24.1%)	0.0290
2 + 3	38 (21.9%)	87 (50.0%)	
ER status, n (%)			
Negative	2 (1.2%)	11 (6.3%)	0.3698
Positive	43 (24.7%)	118 (67.8%)	
PR status, n (%)			
Negative	10 (5.8%)	48 (27.6%)	0.0663
Positive	35 (20.1%)	81 (46.5%)	
Her2 status, n (%)			
Negative	45 (25.9%)	128 (73.5%)	0.5536
Positive	0 (0.0%)	1 (0.6%)	
Ki-67 LI,† (%)	4.3 ± 0.7	6.0 ± 0.8	0.2672

†Data are presented as mean ± SEM. All other values represent the number of the cases and percentage. ‡Data regarding the tumor size were available in the cohorts of Tohoku University Hospital and Kosai Hospital. 17βHSD5, 17β-hydroxysteroid dehydrogenase type 5; 5αRed1, 5α-reductase type 1; ER, Estrogen receptor; Her2, human epidermal growth factor receptor 2; LI, labeling index; PR, Progesterone receptor.

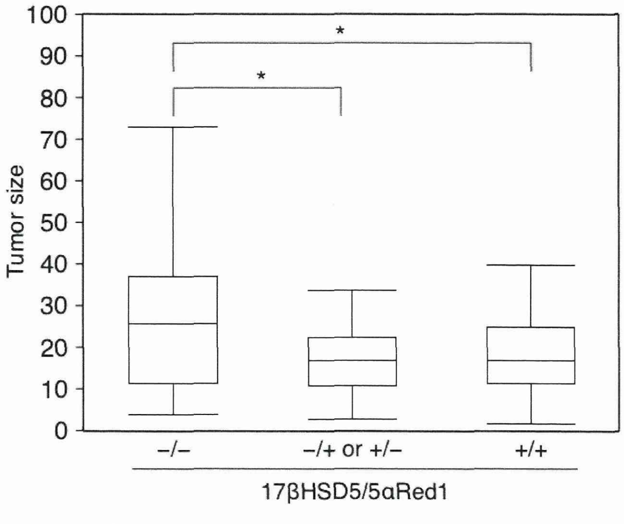

Fig. 2. Tumor size was inversely correlated with the status of androgenic enzymes in androgen receptor-positive invasive lobular carcinoma cases. Data of tumor size were available from a subset of breast cancer patients treated at Tohoku University Hospital or Tohoku Kosai Hospital. *P < 0.05. 17βHSD5, 17β-hydroxysteroid dehydrogenase type 5; 5αRed1, 5α-reductase type 1.

cytoplasm of carcinoma cells (Fig. 4b). The number of 17βHSD2-positive cases was significantly higher in ILC than in IDC despite nearly the same status of hormone receptors between ILC and IDC cases examined (Fig. 4c).

We then evaluated the status of 17βHSD2 in ILC cases using immunohistochemistry in order to analyze the correlation between androgenic enzymes and 17βHSD2 in AR-positive ILC cases. Among patients examined in this study, 101 cases (58.0%) were classified as positive for 17βHSD2. The status of 17βHSD2 was significantly correlated with that of androgenic enzymes (Fig. 4d). The associations between the status

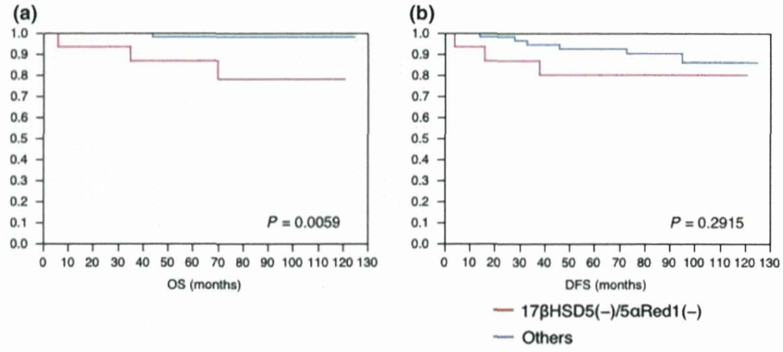

Fig. 3. Absence of intratumoral androgenic enzymes was significantly correlated with adverse clinical outcome in breast cancer patients. Clinical information regarding overall survival (OS) and disease-free survival (DFS) was available for cases from Tohoku University Hospital and Tohoku Kosai Hospital. Only patients with survival data greater than 5 years (surgical date before 2008) were included in this study. The OS (a) and DFS (b) of these patients were analyzed according to the status of intratumoral androgenic enzymes 17β-hydroxysteroid dehydrogenase type 5 (17βHSD5) and 5α-reductase type 1 (5αRed1) using the Kaplan–Meier method (n = 72).

of 17βHSD2 and clinicopathological parameters of the patients are summarized in Table 4. Stage and tumor size were inversely correlated with the status of 17βHSD2 (P = 0.0049 and 0.0299, respectively), and the mean tumor size was also lower in the 17βHSD2-positive group (Fig. 4e). Nuclear grade of the cases examined was also correlated with the status of 17βHSD2 (P = 0.0110).

Discussion

This study showed the presence of AR in almost all cases of ILC (97.8%) and the presence of androgen synthesizing enzymes (17βHSD5, 61.8%; 5αRed1, 53.4%) in the great majority of carcinomas. The prevalence of AR-positive cases in ILC is consistent with that of previously reported studies,[8,12] but the presence of androgenic enzymes in a significant proportion of ILCs represents an entirely novel finding. The high prevalence of AR in ILC excluded a direct assessment of the correlation between AR and clinicopathological characteristics of the patients but the prevalence of androgen synthesizing enzymes, which were detected in approximately 50% of the patients examined, allowed us to examine their effects on clinical outcome of the ILC patients in this study. Results of this particular analysis indicated that the tumors enriched in androgenic pathways (AR-positive/enzyme-positive) were strongly associated with smaller tumor sizes and better clinical outcomes. However, the association between the intratumoral status of AR/enzymes and tumor cell proliferation in ILC could not be assessed due to the inherently low levels of cell proliferation in ILC tissues.[1,31] This aside, the overall findings of our study suggest the importance of androgen signaling and synthesizing enzymes in determining the clinical outcome of ILC patients.

The importance of AR in influencing tumor biology has not been previously evaluated in a large and exclusively ILC cohort. In previous studies examining IDC or mixed IDC/ILC cohorts the expression of AR in cancer tissues has been mostly reported to be associated with a relatively favorable clinical outcome in both ER-positive[15,32] and ER-negative cancers.[33] Results of *in vitro* studies reported in ER-positive breast carcinoma cell lines have also indicated the potent androgen DHT as an inhibitor of breast carcinoma cell proliferation,[15–18] although the underlying molecular biology of the AR in ER-negative cancers is only starting to be elucidated.[13,14] The majority of ILC patients are ER-positive (e.g. 92.7% in this study) and it is reasonable to hypothesize that, as in IDC, AR

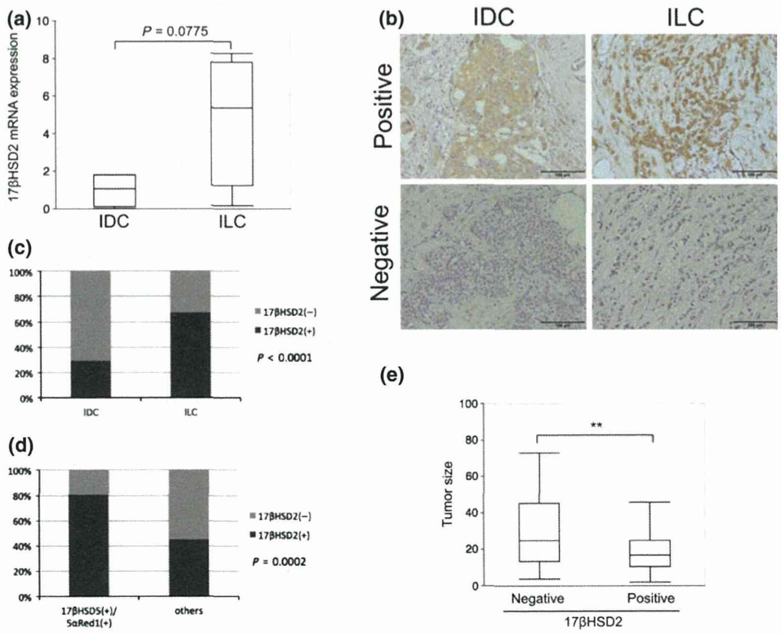

Fig. 4. Status of 17β-hydroxysteroid dehydrogenase type 2 (17βHSD2) immunoreactivity was significantly higher in invasive lobular carcinoma (ILC) than in invasive ductal carcinoma (IDC) and inversely associated with tumor size in ILC cases. (a) 17βHSD2 mRNA expression in four IDC and four ILC cases was analyzed using RT^2 quantitative PCR. (b) Representive illustrations of 17βHSD2 positive (left) and negative (right) cases in IDC (top) and ILC (bottom). 17βHSD2 was localized in the cytoplasm of carcinoma cells. Scale bar = 100 μm. (c) 17βHSD2 immunoreactivity in ILC and IDC specimens. (d) Correlation between 17βHSD2 and androgenic enzymes in androgen receptor-positive ILC cases. (e) Tumor size was inversely correlated with 17βHSD2 expression. Data of tumor size were available in a subset of breast cancer patients treated at Tohoku University Hospital or Tohoku Kosai Hospital. **$P < 0.01$. 17βHSD5, 17β-hydroxysteroid dehydrogenase type 5; 5αRed1, 5α-reductase type 1.

Table 4. Associations between the 17β-hydroxysteroid dehydrogenase type 2 (17βHSD2) expression and clinicopathological parameters in androgen receptor (AR)-positive invasive lobular carcinoma (ILC) cases (n = 174)

	17βHSD2		*P*-value
	Negative n = 73 (42.0%)	Positive n = 101 (58.0%)	
Age,† (years)	56.6 ± 1.5	57.9 ± 1.1	0.4722
Menopausal status, n (%)			
Premenopausal	33 (19.0%)	35 (20.1%)	0.1592
Postmenopausal	40 (23.0%)	66 (37.9%)	
Stage, n (%)			
1	22 (12.6%)	52 (29.9%)	0.0049
2 + 3	51 (29.3%)	49 (28.2%)	
Tumor size,‡ n (%)			
<20 mm	16 (13.0%)	49 (39.9%)	
≥20 mm	25 (20.3%)	33 (26.8%)	0.0299
Nuclear grade, n (%)			
1	28 (16.1%)	21 (12.1%)	0.0110
2 + 3	45 (25.8%)	80 (46.0%)	
ER status, n (%)			
Negative	4 (2.3%)	9 (5.2%)	0.3956
Positive	69 (39.6%)	92 (52.9%)	
PR status, n (%)			
Negative	24 (13.8%)	34 (19.5%)	0.9135
Positive	49 (28.2%)	67 (38.5%)	
Her2 status, n (%)			
Negative	73 (41.9%)	100 (57.5%)	0.3939
Positive	0 (0.0%)	1 (0.6%)	
Ki-67 LI,† (%)	5.5 ± 0.9	5.6 ± 0.9	0.9847

†Data are presented as mean ± SEM. All other values represent the number of the cases and percentage. ‡Data regarding the tumor size were available in the cohorts of Tohoku University Hospital and Kosai Hospital. ER, Eestrogen receptor; Her2, human epidermal growth factor receptor 2; LI, labeling index; PR, progesterone receptor.

has protective effects, possibly more so in ILC cases given their more abundant ER content and more favorable clinical outcome compared to IDC.

The similarities between IDC and ILC are also apparent in a comparison of the normal lobules as compared to ILC areas. The present study showed increased numbers of AR-positive carcinoma cells alongside a decrease in androgenic enzymes detected in ILC compared to non-neoplastic adjacent lobules of the same breast tissue. Higher expression of AR in ILC than in non-neoplastic lobules is also consistent with the reported findings in IDC, that is, higher than in adjacent non-neoplastic ducts,[13] as is the association of AR and enzymes with clinical outcome of the patients[14,25] and the decrease of androgenic enzymes with cancer progression,[29] although changes between non-neoplastic condition and carcinoma initiation are less clear.[34] In addition, the inverse association of PR, commonly considered an estrogen-dependent gene, and androgenic enzymes in ILC detected in our present study suggests that, as in IDC, AR actions may disrupt estrogen signaling in ILC. In non-human primates, AR expression in both lobular and ductal tissues showed similar patterns of prevalence over the reproductive cycle[35] suggesting AR expression in lobules and ducts may be regulated in the same fashion, but this awaits further investigation for clarification.

It has been reported that *17βHSD2* is one of the most important androgen-induced genes in IDC,[27] playing pivotal roles in the putative protective effects of androgen in breast cancer patients. As an enzyme that converts estradiol to estrone or testosterone to androstenedione in breast cancer tissues, 17βHSD2 was reported to be significantly associated with better recurrence-free survival in breast cancer patients.[36] In addition, a significantly inverse correlation was reported between 17βHSD2 expression and intratumoral estradiol concentration, suggesting that this enzyme plays important roles as an estrogen-inactivating enzyme. In this study, we showed a higher expression of 17βHSD2 in ILC than in IDC tissues and an association between androgen pathways and 17βHSD2 in ILC tissues, indicating an androgenic induction of 17βHSD2 in ILC compared to IDC. The potential protective effect of 17βHSD2 in ILC cancers was evaluated by examining the correlations between the status of 17βHSD2 and clinicopathological factors of the cases examined. Results indicated that 17βHSD2 status was inversely correlated with the majority of adverse clinical factors, indicating that 17βHSD2 status of carcinoma cells was associated with less aggressive phenotypes of ILC. This is consistent with the suggestion that androgens may act, at least partially, through the upregulation of 17βHSD2 and thus modify the balance of estrogen production in ILC tissues, although further investigations are required for clarification.

Invasive lobular carcinoma cases are reported to express aromatase at similar or greater levels than IDC.[37] While controversies still exist as to the potential efficacy of AIs in ILC patients,[38,39] Metzger *et al.*[40] suggested that AI treatment was especially effective in ILC patients. This is interesting with regard to the findings of our study as one of the well documented consequences of aromatase inhibition is a shift in the equilibrium of androgens and estrogens towards a greater abundance of androgens and androgen signaling.[26–28] Results of our present study indicated a protective effect of androgens potentiated by alterations of local intracrine signaling and subsequent favoring androgen over estrogen signaling in ILC at a level at least comparable with that of IDC. Therefore, AIs should be at least as effective in ILC patients, especially given the high levels of ER- and AR-positive cases observed in ILC samples.

In this study, we showed that androgen-producing enzymes were involved in tumor suppressive roles of androgens in AR-enriched ILC tissues. In addition, we established the significant correlation of androgen-producing enzymes with 17βHSD2, one of the markers of androgenic actions in breast carcinoma cells, implying that 17βHSD2 reflects intratumoral androgenic actions and acts as suppressor of estrogenic actions. These results could be related to the relatively low proliferative status of carcinoma cells in ILC despite more abundant ER and the presence of intratumoral aromatase, and suggests further benefit of AI treatment against ILC by accumulation of intratumoral androgens.

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Disclosure Statement

The authors have no conflicts of interest.

Abbreviations

17βHSD2	17β-hydroxysteroid dehydrogenase type 2
17βHSD5	17β-hydroxysteroid dehydrogenase type 5
5αRed1	5α-reductase type 1
AI	aromatase inhibitor
AR	androgen receptor
DFS	disease-free survival
DHT	dihydrotestosterone
ER	estrogen receptor
Her2	human epidermal growth factor receptor 2
IDC	invasive ductal carcinoma
ILC	invasive lobular carcinoma
OS	overall survival
PR	progesterone receptor
qPCR	quantitative PCR

References

1 Arpino G, Bardou VJ, Clark GM, Elledge RM. Infiltrating lobular carcinoma of the breast: tumor characteristics and clinical outcome. *Breast Cancer Res* 2004; **6**: R149–56.
2 Bharat A, Gao F, Margenthaler JA. Tumor characteristics and patient outcomes are similar between invasive lobular and mixed invasive ductal/lobular breast cancers but differ from pure invasive ductal breast cancers. *Am J Surg* 2009; **198**: 516–9.
3 Li CI, Anderson BO, Porter P, Holt SK, Daling JR, Moe RE. Changing incidence rate of invasive lobular breast carcinoma among older women. *Cancer* 2000; **88**: 2561–9.
4 Biglia N, Mariani L, Sgro L, Mininanni P, Moggio G, Sismondi P. Increased incidence of lobular breast cancer in women treated with hormone replacement therapy: implications for diagnosis, surgical and medical treatment. *Endocr Relat Cancer* 2007; **14**: 549–67.
5 Korkola JE, DeVries S, Fridlyand J *et al.* Differentiation of lobular versus ductal breast carcinomas by expression microarray analysis. *Cancer Res* 2003; **63**: 7167–75.
6 Zhao H, Langerød A, Ji Y *et al.* Different gene expression patterns in invasive lobular and ductal carcinomas of the breast. *Mol Biol Cell* 2004; **15**: 2523–36.
7 Turashvili G, Bouchal J, Baumforth K *et al.* Novel markers for differentiation of lobular and ductal invasive breast carcinomas by laser microdissection and microarray analysis. *BMC Cancer* 2007; **7**: 55.
8 Rakha EA, El-Sayed ME, Powe DG *et al.* Invasive lobular carcinoma of the breast: response to hormonal therapy and outcomes. *Eur J Cancer* 2008; **44**: 73–83.
9 Wasif N, Maggard MA, Ko CY, Giuliano AE. Invasive lobular vs. ductal breast cancer: a stage-matched comparison of outcomes. *Ann Surg Oncol* 2010; **17**: 1862–9.
10 Moinfar F, Okcu M, Tsybrovskyy O *et al.* Androgen receptors frequently are expressed in breast carcinomas: potential relevance to new therapeutic strategies. *Cancer* 2003; **98**: 703–11.
11 Ogawa Y, Hai E, Matsumoto K *et al.* Androgen receptor expression in breast cancer: relationship with clinicopathological factors and biomarkers. *Int J Clin Oncol* 2008; **13**: 431–5.
12 Riva C, Dainese E, Caprara G *et al.* Immunohistochemical study of androgen receptors in breast carcinoma. Evidence of their frequent expression in lobular carcinoma. *Virchows Arch* 2005; **447**: 695–700.
13 Hickey TE, Robinson JLL, Carroll JS, Tilley WD. Minireview: the androgen receptor in breast tissues: growth inhibitor, tumor suppressor, oncogene? *Mol Endocrinol* 2012; **26**: 1252–67.
14 McNamara KM, Yoda T, Takagi K, Miki Y, Suzuki T, Sasano H. Androgen receptor in triple negative breast cancer. *J Steroid Biochem Mol Biol* 2013; **133**: 66–76.
15 Peters AA, Buchanan G, Ricciardelli C *et al.* Androgen receptor inhibits estrogen receptor-alpha activity and is prognostic in breast cancer. *Cancer Res* 2009; **69**: 6131–40.
16 Andò S, De Amicis F, Rago V *et al.* Breast cancer: from estrogen to androgen receptor. *Mol Cell Endocrinol* 2002; **193**: 121–8.
17 Lapointe J. Role of the cyclin-dependent kinase inhibitor p27Kip1 in Androgen-Induced Inhibition of CAMA-1 breast cancer cell proliferation. *Endocrinology* 2001; **142**: 4331–8.
18 Poulin R, Baker D, Labrie F. Androgens inhibit basal and estrogen-induced cell proliferation in the ZR-75-1 human breast cancer cell line. *Breast Cancer Res Treat* 1988; **12**: 213–25.
19 Lanzino M, De Amicis F, McPhaul MJ, Marsico S, Panno ML, Andò S. Endogenous coactivator ARA70 interacts with estrogen receptor alpha (ERalpha) and modulates the functional ERalpha/androgen receptor interplay in MCF-7 cells. *J Biol Chem* 2005; **280**: 20421–30.
20 Hu R, Dawood S, Holmes MD *et al.* Androgen receptor expression and breast cancer survival in postmenopausal women. *Clin Cancer Res* 2011; **17**: 1867–74.
21 Suzuki T, Miki Y, Nakamura Y *et al.* Sex steroid-producing enzymes in human breast cancer. *Endocr Relat Cancer* 2005; **12**: 701–20.
22 Labrie F, Luu-The V, Labrie C *et al.* Endocrine and intracrine sources of androgens in women: inhibition of breast cancer and other roles of androgens and their precursor dehydroepiandrosterone. *Endocr Rev* 2003; **24**: 152–82.
23 Recchione C, Venturelli E, Manzari A, Cavalleri A, Martinetti A, Secreto G. Testosterone, dihydrotestosterone and oestradiol levels in postmenopausal breast cancer tissues. *J Steroid Biochem Mol Biol* 1995; **52**: 541–6.
24 Suzuki T, Miki Y, Takagi K *et al.* Androgens in human breast carcinoma. *Med Mol Morphol* 2010; **43**: 75–81.
25 Suzuki T, Miki Y, Moriya T *et al.* 5Alpha-reductase type 1 and aromatase in breast carcinoma as regulators of in situ androgen production. *Int J Cancer* 2007; **120**: 285–91.
26 Takagi K, Ishida T, Miki Y *et al.* Intratumoral concentration of estrogens and clinicopathological changes in ductal carcinoma in situ following aromatase inhibitor letrozole treatment. *Br J Cancer* 2013; **109**: 100–8.
27 Takagi K, Miki Y, Nagasaki S *et al.* Increased intratumoral androgens in human breast carcinoma following aromatase inhibitor exemestane treatment. *Endocr Relat Cancer* 2010; **17**: 415–30.
28 Chanplakorn N, Chanplakorn P, Suzuki T *et al.* Increased 5α-reductase type 2 expression in human breast carcinoma following aromatase inhibitor therapy: the correlation with decreased tumor cell proliferation. *Horm Cancer* 2011; **2**: 73–81.
29 Shibahara Y, Miki Y, Sakurada C *et al.* Androgen and androgen-metabolizing enzymes in metastasized lymph nodes of breast cancer. *Hum Pathol* 2013; **44**: 2338–45.
30 Verma MK, Miki Y, Abe K *et al.* Intratumoral localization and activity of 17β-hydroxysteroid dehydrogenase type 1 in non-small cell lung cancer: a potent prognostic factor. *J Transl Med* 2013; **11**: 167.
31 Silverstein MJ, Lewinsky BS, Waisman JR *et al.* Infiltrating lobular carcinoma. Is it different from infiltrating duct carcinoma? *Cancer* 1994; **73**: 1673–7.
32 Bryan RM, Mercer RJ, Bennett RC, Rennie GC, Lie TH, Morgan FJ. Androgen receptors in breast cancer. *Cancer* 1984; **54**: 2436–40.
33 McNamara KM, Yoda T, Miki Y *et al.* Androgenic pathway in triple negative invasive ductal tumors: its correlation with tumor cell proliferation. *Cancer Sci* 2013; **104**: 639–46.
34 Shibuya R, Suzuki T, Miki Y *et al.* Intratumoral concentration of sex steroids and expression of sex steroid-producing enzymes in ductal carcinoma in situ of human breast. *Endocr Relat Cancer* 2008; **15**: 113–24.
35 Cheng G, Li Y, Omoto Y *et al.* Differential regulation of estrogen receptor (ER)alpha and ERbeta in primate mammary gland. *J Clin Endocrinol Metab* 2005; **90**: 435–44.
36 Gunnarsson C, Olsson BM, Stål O. Abnormal expression of 17beta-hydroxysteroid dehydrogenases in breast cancer predicts late recurrence. *Cancer Res* 2001; **61**: 8448–51.
37 Sasano H. Aromatase and 17 beta-hydroxysteroid dehydrogenase type 1 in human breast carcinoma. *J Clin Endocrinol Metab* 1996; **81**: 4042–6.
38 van de Water W, Fontein DBY, van Nes JGH *et al.* Influence of semi-quantitative oestrogen receptor expression on adjuvant endocrine therapy efficacy in ductal and lobular breast cancer - a TEAM study analysis. *Eur J Cancer* 2013; **49**: 297–304.
39 Montemurro F. Upfront adjuvant aromatase inhibitors in women with lobular breast cancer. *Eur J Cancer* 2013; **49**: 3376–7.
40 Metzger O, Giobbie-Hurder A, Mallon E *et al.* Relative effectiveness of letrozole compared with tamoxifen for patients with lobular carcinoma in the BIG 1-98 trial. *Cancer Res* 2012; **72**(1024): S1–1. Available from: http://cancerres.aacrjournals.org/cgi/content/meeting_abstract/72/24_MeetingAbstracts/S1–1.

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The Perioperative Educational Program for Improving Upper Arm Dysfunction in Patients with Breast Cancer: A Controlled Trial

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Most patients who undergo breast cancer surgery suffer from impairment of upper extremity function. In this study, we investigated the effectiveness of a perioperative educational program for improving upper arm dysfunction in patients with breast cancer. This longitudinal controlled study was conducted between January 2010 and July 2012. Participants comprised 149 patients with primary breast cancer before operation, allocated to intervention and control groups. Intervention comprised a 3-month educational program on monitoring arm function and exercises for preventing shoulder dysfunction and lymphedema. The control group received routine care from on-site staffs. Of the 149 patients analyzed, 69 underwent axillary lymph node dissection (ALND), and 80 underwent sentinel lymph node biopsy (SLNB). The intervention group included 39 patients with ALND and 51 patients with SLNB, while the control group included 30 patients with ALND and 29 patients with SLNB. Arm girth, shoulder range of motion (ROM), and grip strength were measured before surgery and at 1 week, 1 month and 3 months postoperatively. Self-reported questionnaires, the Subjective Perception of Post-Operative Functional Impairment of the Arm (SPOFIA) and the Disabilities of the Arm, Shoulder and Hand (DASH), were administered at the same time points. Among the variables examined, only SPOFIA and grip strength were significantly improved in the intervention group with ALND. In contrast, the perioperative educational program caused no significant improvement for the patients who underwent the surgery with SLNB. Thus, the present program improves the postoperative upper arm function and discomfort in breast cancer patients who undergo surgery with ALND.

Keywords: breast cancer; controlled trial; education; surgery; upper arm dysfunction
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Introduction

Recently, the standardization of multimodal therapy and sentinel lymph node biopsy (SLNB) has resulted in more conservative treatment of breast cancer, compared to the surgical treatment with axillary lymph node dissection (ALND). However, invasive surgery is unavoidable in cases of more malignant cancer involving axillary lymph node metastases. Impaired upper extremity function following surgery for breast cancer is a particular complication of ALND. Post-surgical morbidity has also been reported in SLNB treatment groups, although its frequency is lower than in patients undergoing ALND (Ashikaga et al. 2010). Assessment and support of upper extremity function following breast cancer surgery are therefore applicable not just after ALND, but also after SLNB. The Upper Extremity Rehabilitation Guideline recommends 6-8 weeks of shoulder joint range exercise after breast cancer surgery and 1 year of postoperative follow-up (Harris et al. 2012). However, numbness, pain, swelling, and limitation of arm movement occurred in 8-35% of 330 breast cancer patients for 2-5 years postoperatively (Warmuth et al. 1998), and chronic symptoms have been reported (Hack et al. 1999; Voogd et al. 2003; Macdonald et al. 2005). In a cross-sectional survey that we conducted, 85.3% of 150 breast cancer patients experienced at least one of swelling, pain, decreased shoulder range of motion (ROM), numbness, reduced muscle strength in the arm, or a feeling of pulling in the skin of the arm up to 1 year postoperatively, with a greater loss to quality of life (QOL) in those patients experiencing such symptoms (Sato and Kuroda 2008). Some patients have reported problems with writing, and actions such as opening or closing jars in daily activities, and work can be affected due to weakened grip strength after surgery.

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As hospital stays become increasingly shorter, there is a need for systematic forms of support such as ongoing rehabilitation and individual counseling in support of breast cancer patients after they leave the hospital.

As one example of an intervention study for preventing or improving impaired upper extremity function after breast cancer surgery, Wyatt and Friedman (1996) developed and demonstrated the efficacy of a support program based on a holistic framework for QOL, encompassing strategies for preventing impaired upper extremity function and lymphedema, physical care, mental health, and family involvement. This program showed effectiveness in self-care, emotional well-being, physical well-being, and social/family well-being. However, the effectiveness of the program may differ in Japan, as hospital stays are longer and the home-care system is relatively underdeveloped. In addition, most rehabilitation for postoperative impairment of upper extremity function has been developed to recover shoulder ROM and minimize lymphedema (Box et al. 2002; Harris et al. 2012). No studies have yet reported the tracking of changes in upper extremity function over time to assess the effects of a program that was not limited to lymphedema or shoulder ROM and that was suitable for impaired upper extremity function in Japanese breast cancer patients.

The objective of this study was to investigate the effectiveness of short-term intervention for up to 3 months postoperatively using a program intended to prevent or improve impaired upper extremity function after breast cancer surgery, as assessed on the basis of changes over time in upper extremity function with ALND or SLNB.

Methods

Study design

This study was a controlled trial. Patients were allocated to the intervention or control group according to their wishes after receiving full information about the study protocols and providing informed consent.

Study participants

Breast cancer patients who had yet to undergo surgery were recruited in Tohoku University Hospital between January 2010 and April 2012. Data collection ended in July 2012. The inclusion criteria were: 1) age $\geq$ 20 years; 2) ability to answer a self-administered questionnaire and no history of diagnosis or treatment for any mental illness; and 3) provision of written informed consent to participate in the survey as per the protocol approved by the Ethics Committee of the Research Department at Tohoku University Graduate School of Medicine. Patients with bilateral breast cancer or recurrence were excluded.

Intervention

The theoretical framework of the intervention program was based on the University of California, San Francisco (UCSF) symptom management model (The University of California, San Francisco School of Nursing Symptom Management Faculty Group 1994), which was developed on the basis of self-care theory. The UCSF Model defines a symptom as a subjective experience reflecting changes in biopsychosocial functioning, sensations, or cognition of an individual, and was designed to produce an integrated approach for symptom management through a comprehensive grasp of patient and family symptom experience, symptom management strategies, and outcomes. This framework was intended to guide training and practice, as well as research on symptoms. The program was created to implement educational intervention for the prevention or improvement of postoperative swelling, pain, decreased shoulder ROM, numbness, reduced muscle strength of the arm, or feeling of pulling in the skin of the arm in breast cancer patients, to transform knowledge of the sciences of ecology and health as well as self-care strategies, and to change the symptoms of impaired upper extremity function and QOL. The appropriateness of the contents of the program for cancer nursing researchers, healthcare professionals, and breast cancer patients has been reviewed (Sato 2012a).

The mechanisms and causes of symptom development were explained prior to surgery, and techniques to prevent or improve impairment of upper extremity function potentially occurring as a result of the surgical procedure undergone by the subject were explained after surgery until the patient was discharged from hospital. Methods of arm monitoring, exercises for preventing restricted shoulder ROM or lymphedema, and massaging methods were also demonstrated and implemented with the subject until learned. Patients were asked to incorporate such knowledge and skills in their activities and put them into practice after leaving hospital. During the surveys at 1 and 3 months postoperatively, patients were assessed for upper extremity function, symptom experience, strategies, and outcomes, and individual support was provided to assist and enhance symptom management. Patients in the control group received routine care from on-site staff and were informed of the results of upper extremity function determined in the survey.

Measurement

We measured arm girth, shoulder ROM and grip strength, and administered the Subjective Perception of Post-Operative Functional Impairment of the Arm (SPOFIA) questionnaire (tested for reliability and validity by Sato (2008)), and the Japanese Society for Surgery of the Hand (JSSH) version of the Disabilities of the Arm, Shoulder and Hand (DASH) questionnaire (tested for reliability and validity by Jester et al. (2005)) (Table 1) at the hospital admission preoperatively, the day after drain removal (approximately 1 week postoperatively), and at 1 and 3 months postoperatively.

Objective outcomes, including arm girth, shoulder ROM and grip strength, were measured by a specialist with standard methods. Arm girth measurements were taken at 2 points, 10 cm distal to the lateral epicondyle (forearm arm girth), and 15 cm proximal to the lateral epicondyle (upper arm girth) (Kissin et al. 1986; Ivens et al. 1992). The difference between arm girths on the affected and normal sides was calculated. Shoulder ROM measured shoulder flexion, shoulder abduction, and horizontal shoulder extension, and differences between normal and affected sides were calculated. Grip strength measured using a dynamometer, and the difference between normal and affected sides was calculated.

The SPOFIA uses a 2-point assessment (yes, 1 point; no, 0 points) of 15 items related to swelling, pain, decreased shoulder ROM, numbness, reduced muscle strength of the arm, and pulling feeling in the skin of the arm skin. Higher SPOFIA scores indicate a greater perception of postoperative impairment in upper extremity

Table 1. Questionnaire items.

SPOFIA (15 items)
1. The forearm is swollen (from elbow to fingertip)
2. The upper arm is swollen (from elbow to shoulder)
3. The arm is heavy
4. The arm is tired
5. Pain when clothes touch the arm
6. Pain when moving the arm
7. Pain even if not moving the arm.
8. Cannot raise the arm on the operated side straight forward to the level of the ear without bending the elbow
9. Cannot raise the arm on the operated side sideways to the level of the ear without bending the elbow
10. Cannot raise the arm on the operated side sideways and backwards without bending the elbow
11. Partial numbness when touching
12. Feeling of numbness
13. Weakness when lifting things
14. Weakness when gripping things
15. Pulling feeling of arm skin when lifting the arm

DASH (30 items)
1. Open a tight or new jar
2. Write
3. Turn key
4. Prepare a meal
5. Push open a heavy door
6. Place an object on a shelf above your head
7. Do heavy household chores (e.g., wash walls, wash floors)
8. Perform gardening or yard work
9. Make a bed or lay out the bedding
10. Carry a shopping bag or briefcase
11. Carry a heavy object (> 5 kg)
12. Change a light bulb overhead
13. Wash or dry your hair
14. Wash your back
15. Put on a pullover sweater
16. Use a knife to cut food
17. Perform recreational activities that require little effort (e.g. card playing, knitting, play go, play shogi etc.)
18. Perform recreational activities involving some force or action through the arm, shoulder or hand (e.g., golf, tennis, playing catch, hammering, etc.)
19. Perform recreational activities in which you move your arm freely (e.g., playing Frisbee, badminton, etc.)
20. Manage transportation needs
21. Perform sexual activities
22. During the past week, to what extent has your arm, shoulder or hand interfered with your normal social activities with family, friends, neighbors or groups?
23. During the past week, were you limited in your work or other daily activities as a result of your arm, shoulder or hand problem?
24. Arm, shoulder or hand pain
25. Arm, shoulder or hand pain when performing a specific activity
26. Tingling (pins and needles) in the arm, shoulder or hand
27. Weakness in the arm, shoulder or hand
28. Stiffness in the arm, shoulder or hand
29. During the past week, how much difficulty have you had sleeping because of pain in your arm, shoulder or hand?
30. I feel less capable, less confident or less useful because of my arm, shoulder or hand problem

function. Cronbach's alpha coefficient for this scale was 0.76.

The DASH is a standardized questionnaire that evaluates impairments and limitations to activity, in addition to restrictions on participation in both leisure activities and work. Response options range from 1 to 5, as follows: 1, no difficulty; 2, mild difficulty; 3, moderate difficulty; 4, severe difficulty; and 5, inability. The DASH produces scores between 0 and 100 for each module, with a high DASH score indicating severe disability (Jester et al. 2005). Cronbach's alpha coefficient for this scale was 0.79.

Demographic characteristics (age, marital status, occupation, child care, caring for an older relative, disease severity, arm dominance, shoulder problems before operation) were self-reported at baseline. History of disease including type of surgery, level of axillary lymph node dissection, and adjuvant treatment were taken from medical records.

The information sheet was read to the patient, upper extremity functions were measured, and the patient was given the questionnaire form and told how to complete it. The questionnaire was then collected after the patient had completed it. When the questionnaire was collected, the patient was asked to check and ensure that all questions had been answered. For the surveys at 1 and 3 months postoperatively, the interviewers showed up on the days that subjects were scheduled to visit, and surveys were conducted in accordance with the schedules of staff members responsible for the examination and care of the subjects. The consent of subjects was also confirmed.

Statistics

The intervention and control groups were compared according to performance of ALND and SLNB. Demographic variables were compared using the Mann-Whitney U test or Fisher's exact test, and changes over time in upper extremity function were compared by two-way repeated-measures analysis of variance. Statistical analysis was performed using PASW Statistics for Windows version 21.0 (SPSS, Tokyo, Japan). The significance level was ≤ 5%.

Results

A total of 162 patients participated this study and were allocated to an intervention group (n = 96) and a control group (n = 66). In the intervention group, a total of 6 patients dropped out due to loss to follow-up, changing hospital, loss of interest or lack of time. In the control group, a total of 7 patients dropped out due to loss to follow-up, changing hospital, loss of interest or lack of time. As a result, 149 patients completed the study (Fig. 1). Of the 149 patients analyzed, 69 underwent ALND, and 80 underwent SLNB. These included 39 patients in the ALND intervention group, 30 patients in the ALND control group, 51 patients in the SLNB intervention group and 29 patients in the SLNB control group.

Table 2 shows the characteristics of patients. No significant differences in demographic or disease characteristics were seen between the ALND and SLNB in the intervention group and ALND and SLNB in the control group.

Table 3 shows changes of variables over time in patients with ALND and Table 4 shows the same in patients with SLNB. No significant differences in arm girth, shoulder ROM or DASH were seen between groups with ALND. Significant differences in change in SPOFIA score over time were noted between the ALND intervention group and the ALND control group (F value = 3.34; p = .02). These results suggest a significant improvement over time in SPOFIA score in the intervention group compared to the control group. In ALND groups, the mean difference in grip strength between normal and affected sides in the intervention group did not differ significantly from baseline to 3 months postoperatively, with a low value at 3 months postoperatively compared to baseline. A significant difference over time in the difference in mean grip strength was seen between normal and affected sides in both intervention and control groups (F value = 2.77; p = .04), indicating significantly improved grip strength over time in the intervention group compared to the control group.

Table 4 shows a comparison of changes over time in arm function of patients between the intervention and control groups with SLNB. No significant differences in arm girth, shoulder ROM, grip strength, SPOFIA or DASH were identified between SLNB groups.

Discussion

In this survey, the effectiveness of short-term intervention based on the Program for Preventing and Improving Postoperative Functional Impairment of the Upper Limbs in Breast Cancer Patients was examined on the basis of

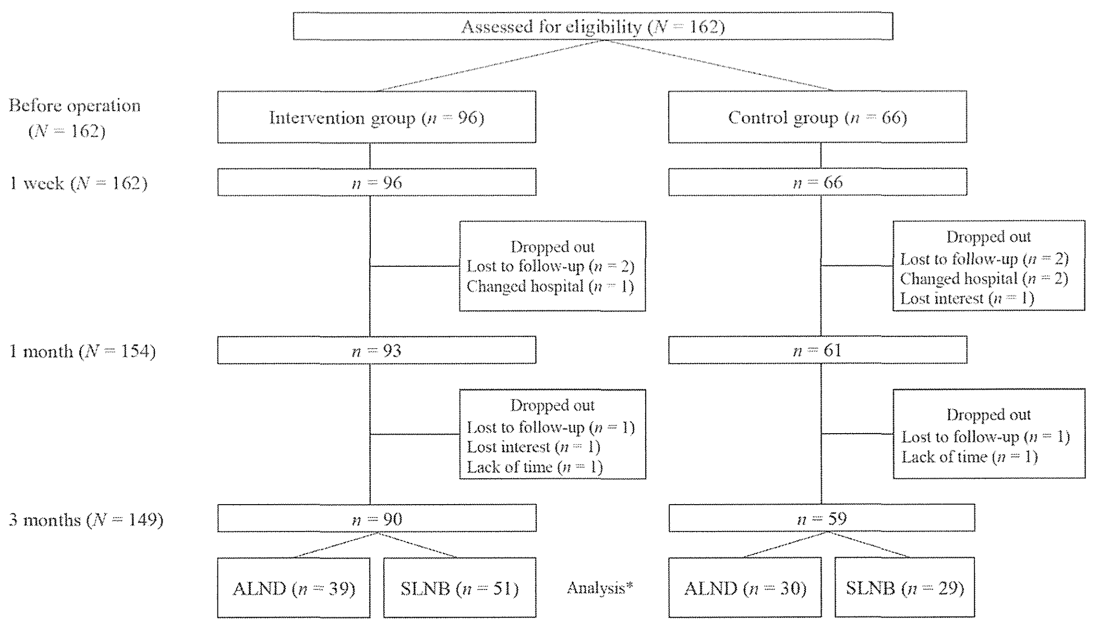

Fig. 1. Flow-chart showing experimental process.
*The intervention and control groups were compared according to performance of axillary lymph node dissection (ALND) and sentinel lymph node biopsy (SLNB).

Table 2. Comparison of patients between groups.

$N = 149$

	ALND (n = 69)			SLNB (n = 80)		
	Intervention	Control	p	Intervention	Control	p
	n = 39	n = 30		n = 51	n = 29	
Age, years: mean ± S.D.	52.9 ± 10.1	52.1 ± 12.9	.70	54.3 ± 10.6	53.7 ± 9.5	.91
Marital status, %	87.1	80.0	.51	84.3	82.8	1.0
Occupation, %	35.9	50.0	.32	47.1	37.9	.29
Child care, %	20.5	10.0	.33	11.8	0.0	.08
Caring for an older relative, %	10.3	0.0	.13	7.8	3.4	.65
Disease severity, %						
Stage 0	0.0	6.7		25.5	44.8	
Stage I	7.7	20.0		54.9	48.3	
Stage II	43.6	50.0	.07	17.6	6.9	.22
Stage III	41.0	23.3		2.0	0.0	
Stage IV	7.7	0.0		0.0	0.0	
Type of surgery, %						
Partial mastectomy	43.6	63.3	.15	78.4	75.9	.79
Total mastectomy	56.4	36.7		21.6	24.1	
Level of axillary lymph node dissection, %						
I	23.1	40.0		—	—	
II	59.0	43.3	.30	—	—	
III	17.9	16.7		—	—	
Adjuvant treatment, %						
Radiotherapy	69.2	83.3	.26	58.8	51.7	.64
Chemotherapy, molecular targeting therapy	79.5	86.7	.36	11.8	24.1	.21
Hormone therapy	82.1	63.3	.10	80.4	75.9	.78
Arm dominance, %	56.4	46.7	.47	54.9	55.2	1.0
Shoulder problem before operation, %	20.5	20.0	1.0	29.4	17.2	.29

Age: Mann-Whitney U test; other details: Fisher's exact test.

Table 3. Comparison of changes in arm function over time for patients with ALND.

	Intervention (n = 39)				Control (n = 30)				F	p
	Baseline[a]	1 week[b]	1 month[c]	3 months[d]	Baseline[a]	1 week[b]	1 month[c]	3 months[d]		
	Mean (S.D.)				Mean (S.D.)					
SPOFIA (0-15)	1.1 (1.9)	6.1 (2.7)	4.6 (3.4)	2.5 (1.9)	0.6 (1.0)	6.2 (3.0)	3.4 (2.7)	3.2 (2.6)	3.34	.02*
DASH (0-100)	8.9 (9.9)	24.9 (15.8)	15.0 (10.2)	10.5 (8.7)	6.0 (10.1)	26.8 (18.2)	12.1 (0.1)	10.4 (8.1)	0.94	.38
Upper arm girth	0.5 (1.1)	0.6 (1.1)	0.6 (1.1)	0.6 (1.1)	−0.2 (1.0)	0.4 (1.1)	0.1 (1.0)	−0.1 (1.0)	2.42	.08
Forearm arm girth	0.4 (1.1)	0.4 (1.2)	0.2 (1.2)	0.3 (1.1)	−0.1 (0.8)	0.1 (1.0)	0.1 (1.2)	0.2 (1.2)	0.70	.54
Flexion shoulder	1.2 (8.1)	30.3 (28.8)	11.3 (16.7)	6.4 (9.6)	−2.4 (7.3)	31.8 (30.4)	3.8 (7.2)	2.9 (10.1)	0.93	.38
Abduction shoulder	3.5 (12.3)	30.9 (30.5)	11.4 (14.8)	3.6 (10.5)	−1.6 (9.2)	31.2 (33.3)	3.6 (8.0)	3.0 (10.4)	0.90	.38
Horizontal extension shoulder	0.5 (7.5)	3.6 (5.8)	2.0 (4.4)	0.5 (6.4)	−0.9 (5.7)	1.4 (7.0)	0.7 (6.6)	0.6 (5.3)	0.47	.70
Grip strength	−0.2 (2.9)	0.6 (3.0)	0.2 (1.2)	−0.8 (4.0)	0.9 (2.9)	2.7 (3.9)	0.1 (1.2)	1.2 (3.6)	2.77	.04*

Two-way repeated-measures ANOVA $*p < .05$.

[a]at hospital admission before operation; [b]day after drain removal (approximately 1 week after operation); [c]1 month after operation; [d]3 months after operation.

Table 4. Comparison of changes in arm function over time for patients with SLNB.

	Intervention (n = 51)				Control (n = 29)				F	p
	Baseline[a]	1 week[b]	1 month[c]	3 months[d]	Baseline[a]	1 week[b]	1 month[c]	3 months[d]		
	Mean (S.D.)				Mean (S.D.)					
SPOFIA (0-15)	0.7 (1.4)	3.7 (2.9)	1.7 (2.3)	1.1 (1.7)	0.3 (0.8)	3.2 (2.3)	1.4 (1.7)	1.1 (1.4)	0.28	.81
DASH (0-100)	6.7 (11.6)	23.4 (18.5)	9.5 (13.3)	7.4 (11.8)	4.7 (9.0)	16.6 (16.3)	9.6 (11.3)	5.6 (6.1)	2.13	.14
Upper arm girth	0.1 (1.2)	0.1 (0.9)	0.1 (0.8)	0.0 (0.9)	0.1 (1.0)	0.1 (1.2)	0.1 (1.0)	0.1 (1.3)	0.10	.95
Forearm arm girth	0.1 (1.0)	0.1 (0.9)	0.0 (1.0)	0.1 (1.0)	0.0 (0.9)	0.1 (1.2)	0.0 (1.3)	−0.1 (1.1)	0.66	.57
Flexion shoulder	1.7 (8.1)	18.9 (26.3)	3.9 (9.3)	3.8 (14.8)	2.1 (9.1)	20.1 (26.2)	8.1 (14.4)	2.0 (10.7)	0.52	.57
Abduction shoulder	−0.1 (8.9)	17.8 (28.8)	2.3 (8.5)	3.7 (11.5)	2.1 (8.9)	18.3 (26.8)	7.2 (16.7)	1.3 (12.1)	0.72	.46
Horizontal extension shoulder	1.3 (5.3)	3.0 (8.1)	1.2 (3.8)	0.2 (4.9)	−0.4 (7.0)	2.4 (7.5)	1.2 (6.9)	0.4 (4.9)	0.55	.64
Grip strength	−0.3 (2.5)	1.1 (3.5)	0.3 (2.9)	−0.2 (2.4)	−0.6 (3.8)	2.2 (3.6)	−0.4 (4.6)	−0.2 (3.5)	2.12	.11

Two-way repeated-measures ANOVA.
[a]at hospital admission before operation; [b]day after drain removal (approximately 1 week after operation); [c]1 month after operation; [d]3 months after operation.

changes over time in upper extremity function in 149 patients who had undergone surgery for primary breast cancer. The results thus suggest that this program provides significant improvement in grip strength and subjective perception of impaired upper extremity function in breast cancer patients undergoing ALND, which is significantly more invasive than SLNB. This discussion focuses on the effectiveness of short-term intervention using this program based on the present results.

We first discuss whether the program resulted in effective improvement over time in SPOFIA score. The first reason for improvement in SPOFIA score is the SPOFIA scale. The SPOFIA scale used in this survey encompassed swelling, decreased shoulder ROM, and reduced muscle strength of the arm, which were assessed both objectively and subjectively in this survey, as well as symptoms related to pain, numbness, and pulling feeling in the skin of the arm, which could only be assessed subjectively by patients. Subjective symptom experience is the most important measure in the assessment of physical function (Segerström et al. 1991), and is often considerably distressing to patients (Petrek et al. 2000; Sato 2012b). Avoidance behavior is followed more often by patients who perceive lymphedema (McLaughlin et al. 2008). In this program, differences in measured symptoms and methods of measurement, as well as the presence or absence of perceptions of pain, appeared to be reflected in the subjective assessment of upper extremity function. The second reason for the improvement of SPOFIA may be the appropriate assessment and proper management of symptoms through the discussion between nurses and the patient. Patients in control group generally did not discuss their symptoms with healthcare workers. Patients in the control group were not educated about addressing their symptoms to healthcare workers and they might have thought that symptoms were unable to be managed. According to a fact-finding study on care for impaired upper extremity function following breast cancer surgery in Japan, virtually no patients received treatment for post-mastectomy pain syndrome (PMPS), even though it could be relieved with pharmacotherapy (Yamauchi and Kitahara 2003), and 30% of patients felt unable to broach the subject of PMPS with their doctors. Healthcare professional-based treatment or care systems may not function very well as systems reflecting patient symptom experience. Healthcare professionals must not only have a biological understanding of the postoperative impairment of upper extremity function in breast cancer patients, but also understand and prospectively assess such impaired function as pain that is felt physically and mentally, depending on the connection with social activities and environment. The third reason is the strategy used in the program. This program proposes a strategy in which information on symptom management is provided as needed to breast cancer patients, and symptoms are reported without reservation to healthcare professionals. This strategy may help to alleviate anxiety over symptoms in breast cancer patients and may be applicable to symptom management strategies. The program also incorporates abdominal breathing exercises, and massaging of the upper extremities and areas around the mastectomy wound. These methods may enhance relaxation and circulation, and may improve subjective perception of symptoms.

We now discuss the fact that the grip strength of breast cancer patients in the ALND group who selected the intervention program improved significantly over time compared to that in the control group. Active upper-extremity stretching exercises are recommended to start 1 week after surgery or after the drain is removed and should be continued until full ROM is achieved (Harris et al. 2012). The reason for the lack of any significant difference in shoulder ROM between the intervention and control groups in this program may have been that these upper extremity rehabilitation guidelines are well known and practiced. However, grip strength rehabilitation does not appear to be continued to the same extent as rehabilitation to improve the shoulder ROM. No studies have assessed intervention for facilitating and maintaining recovery from decreased muscle strength, including grip strength. A woman with breast cancer 5 years after ALND whom we had previously interviewed told us that her pen pressure had decreased as a result of her operation, making it difficult to write, and as a result she lost her job as a primary school teacher. Grip strength is used in activities such as writing and opening or closing jars, and is a daily activity function that is as important as shoulder ROM. The present program includes an exercise that begins by gripping a heart-shaped ball with both hands starting on Day 1 after surgery. Continuing this exercise may be a reason for the improvement in grip strength over time.

We will now consider the background against which the ALND group, but not the SLNB group, showed changes over time in SPOFIA score and grip strength intervention in this program. The causes and mechanisms involved in the development of symptoms were explained to the intervention group prior to surgery. The intervention group of the ALND group also received an explanation of the duration of symptoms, symptoms that could potentially develop in the future, activities that should be avoided, and so forth, following individual assessment of the extent of lymph node dissection, neurectomy status, and the like. The importance of making life adjustments based on patient symptom experience, strategies, and outcomes was also discussed starting 1 month after surgery, and support was provided to help patients decide on subsequent symptom strategies. The same strategy was used in the intervention group undergoing SLNB. However, it is assumed that the outcomes are reflective of the lower incidence of SLNB symptoms compared to ALND (Wilke et al. 2006; Ashikaga et al. 2010) as well as differences in the sense of crisis concerning impaired upper extremity function and awareness of the need for modifying daily activities.

DASH score and circumference of the arm showed no significant differences. The DASH used in this survey is a scale for evaluating overall loss of upper extremity function seen in the context of daily activities and environment. The highest DASH score in this survey was 20 out of 100 possible points at 1 week after surgery, indicating a smaller than expected decrease in upper extremity ability. This may have happened because the DASH scale is not limited to upper extremities on the affected side and assesses upper extremity ability on both sides. How this program affects the daily activities of breast cancer survivors should be analyzed in the future, with the inclusion of QOL measurements or qualitative date in addition to DASH measurements. A review of changes over time showed that DASH scores still had not returned to preoperative levels by 3 months after surgery, regardless of differences between ALND and SLNB therapy and whether intervention was used. DASH did not show a significant difference because no patients experienced severe impairment in their life after the operation. The lack of a significant difference in the circumference of the arm between groups may be due to the study period being too short to detect lymphedema. In this survey, the effectiveness of the program was explained on the basis of changes over time in upper extremity function up to 3 months after surgery. However, lymphedema occurs for several years after surgery in relation to activities and environment (Warmuth et al. 1998). A longitudinal survey should be conducted in the future to explore the effectiveness of long-term intervention.

In conclusion, after a 3-month perioperative educational program, SPOFIA and grip strength were significantly improved in the intervention group with ALND. This program may improve the postoperative upper arm function and discomfort in patients who undergo ALND. The long-term effectiveness of the program should be studied in the future.

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Conflict of Interest

The authors declare no conflict of interest.

References

Ashikaga, T., Krag, D.N., Land, S.R., Julian, T.B., Anderson, S.J., Brown, A.M., Skelly, J.M., Harlow, S.P., Weaver, D.L., Mamounas, E.P., Costantino, J.P. & Wolmark, N.; the National Surgical Adjuvant Breast and Bowel Project (NSABP) (2010) Morbidity results from the NSABP B-32 trial comparing sentinel lymph node dissection versus axillary dissection. *J. Surg. Oncol.*, **102**, 111-118.

Box, R.C., Reul-Hirche, H.M., Bullock-Saxton, J.E. & Furnival, C.M. (2002) Shoulder movement after breast cancer surgery: results of a randomised controlled study of postoperative physiotherapy. *Breast Cancer Res. Treat.*, **75**, 35-50.

Hack, T.F., Cohen, L., Katz, J., Robson, L.S. & Goss, P. (1999) Physical and psychological morbidity after axillary lymph node dissection for breast cancer. *J. Clin. Oncol.*, **17**, 143-149.

Harris, S.R., Schmitz, K.H., Campbell, K.L. & McNeely, M.L. (2012) Clinical practice guidelines for breast cancer rehabilitation: syntheses of guideline recommendations and qualitative appraisals. *Cancer*, **118**, 2312-2324.

Ivens, D., Hoe, A.L., Podd, T.J., Hamilton, C.R., Taylor, I. & Royle, G.T. (1992) Assessment of morbidity from complete axillary dissection. *Br. J. Cancer*, **66**, 136-138.

Jester, A., Harth, A., Wind, G., Germann, G. & Sauerbier, M. (2005) Disability of the Arm, Shoulder and Hand (DASH) questionnaire: determining functional activity profiles in patients with upper extremity disorders. *J. Hand Surg. Br.*, **30**, 23-28.

Kissin, M.W., Querci della Rovere, G., Easton, D. & Westbury, G. (1986) Risk of lymphoedema following the treatment of breast cancer. *Br. J. Surg.*, **73**, 580-584.

Macdonald, L., Bruce, J., Scott, N.W., Smith, W.C.S. & Chambers, W.A. (2005) Long-term follow-up of breast cancer survivors with post-mastectomy pain syndrome. *Br. J. Cancer*, **92**, 225-230.

McLaughlin, S.A., Wright, M.J., Morris, K.T., Sampson, M.R., Brockway, J.P., Hurley, K.E., Riedel, E.R. & Van Zee, K.J. (2008) Prevalence of lymphedema in women with breast cancer 5 years after sentinel lymph node biopsy or axillary dissection: patient perceptions and precautionary behaviors. *J. Clin. Oncol.*, **26**, 5220-5226.

Petrek, J.A., Pressman, P.I. & Smith, R.A. (2000) Lymphedema: current issues in research and management. *CA Cancer J. Clin.*, **50**, 292-307.

Sato, F. (2008) Development of an instrument for the evaluation of subjective perception of postoperative functional impairment of the arm in breast cancer survivors. *J. Jpn. Soc. Cancer Nurs.*, **22**, 31-42 (abstract in English, text in Japanese).

Sato, F. (2012a) Development of a program for preventing, and improving postoperative functional impairment of the upper limbs in breast cancer survivors. *Bull. Sch. Health Sci. Tohoku Univ.*, **21**, 65-75 (abstract in English, text in Japanese).

Sato, F. (2012b) Factors related to pain in the arm on the affected side in breast cancer survivors who had undergone surgery within the past year. *J. Jpn. Acad. Health Behav. Sci.*, **27**, 157-170 (abstract in English, text in Japanese).

Sato, F. & Kuroda, Y. (2008) Subjective perception of post-operative functional impairment of the arm and its association with quality of life in breast cancer survivors within the first post-operative year. *J. Jpn. Acad. Nurs. Sci.*, **28**, 28-36 (abstract in English, text in Japanese).

Segerström, K., Bjerle, P. & Nyström, A. (1991) Importance of time in assessing arm and hand function after treatment of breast cancer. *Scand. J. Plast. Reconstr. Surg. Hand Surg.*, **25**, 241-244.

The University of California, San Francisco School of Nursing Symptom Management Faculty Group (1994) A model for symptom management. *Image J. Nurs. Sch.*, **26**, 272-276.

Voogd, A.C., Ververs, J.M.M.A., Vingerhoets, A.J.J.M., Roumen, R.M.H., Coebergh, J.W.W. & Crommelin, M.A. (2003) Lymphoedema and reduced shoulder function as indicators of quality of life after axillary lymph node dissection for invasive breast cancer. *Br. J. Surg.*, **90**, 76-81.

Warmuth, M.A., Bowen, G., Prosnitz, L.R., Chu, L., Broadwater, G., Peterson, B., Leight, G. & Winer, E.P. (1998) Complications of axillary lymph node dissection for carcinoma of the breast: a report based on a patient survey. *Cancer*, **83**, 1362-1368.

Wilke, L.G., McCall, L.M., Posther, K.E., Whitworth, P.W., Reintgen, D.S., Leitch, A.M., Gabram, S.G.A., Lucci, A., Cox, C.E., Hunt, K.K., Herndon, J.E. 2nd. & Giuliano, A.E. (2006) Surgical complications associated with sentinel lymph node biopsy: results from a prospective international cooperative group trial. *Ann. Surg. Oncol.*, **13**, 491-500.

Wyatt, G.K. & Friedman, L.L. (1996) Development and testing of a quality of life model for long-term female cancer survivors. *Qual. Life Res.*, **5**, 387-394.

Yamauchi, K. & Kitahara, M. (2003) Pain and unpleasant sensation after the breast surgery. *J. Breast Cancer*, **18**, 190-195.

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PRECLINICAL STUDY

Tumor-infiltrating CD8+ and FOXP3+ lymphocytes in triple-negative breast cancer: its correlation with pathological complete response to neoadjuvant chemotherapy

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Abstract The anti-tumor immune response was recently reported to play a critical role in the chemotherapeutic sensitivity of breast cancer. Therefore, we investigated the correlation between CD8+ and FOXP3+ tumor-infiltrating lymphocytes and the pathological complete response (pCR) following neoadjuvant chemotherapy (NAC) in triple-negative breast cancer (TNBC), in conjunction with neoangiogenesis, basal and proliferation markers. CD8+ and FOXP3+ lymphocytes were assessed in biopsy specimens by double-staining immunohistochemistry, in combination with immunostaining of vasohibin-1, CD31, EGFR, CK5/6, and Ki-67. Earlier age, pre-menopausal status, smaller tumor size, and high Ki-67 were significantly associated with pCR, as in high CD8+, high CD8+/FOXP3+ ratio, and low vasohibin-1 positive ratio. Multivariate analysis did reveal that a high CD8+/FOXP3+ ratio was a strong predictor of pCR with an odds ratio of 5.32 ($P = 0.005$). High Ki-67 was also significantly associated with pCR ($P = 0.002$). TNBCs with a high CD8+/FOXP3+ ratio and high Ki-67 had the highest pCR rate (70 %) following NAC. However, the pCR rate of the patients with low CD8+/FOXP3+ ratio and low Ki-67 was only 5 %. The pCR rates of a high CD8+/FOXP3+ ratio and low Ki-67 patients and those with a low CD8+/FOXP3+ ratio and high Ki-67 were 24 and 21 %, respectively. TNBCs with a high CD8+/FOXP3+ ratio were more sensitive to anthracycline and taxane-based chemotherapeutic regimens, and the CD8+/FOXP3+ ratio in conjunction with Ki-67 could predict pCR following NAC in TNBC. This predictor may represent a new surrogate for testing the efficacy of investigational agents in the neoadjuvant setting.

Keywords Triple-negative breast cancer · Chemo-sensitivity · pCR · CD8 · FOXP3 · Ki-67

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Introduction

Breast cancer is a heterogeneous disease according to the results of gene expression profiling using microarray analysis [1–3]. Based on the differences in sensitivity to therapeutic drugs and in clinical outcomes, the implementation of personalized medicine is required for treating breast cancer. Triple-negative breast cancer (TNBC), which lacks estrogen receptor (ER), progesterone receptor (PgR) and human epidermal growth factor receptor-2 (HER2) expression, is the breast cancer subtype associated with the worst clinical outcome. Chemotherapy with anthracyclines and taxanes usually constitute the backbone of the treatment of this aggressive subtype, and a pathological complete response (pCR) following neoadjuvant chemotherapy (NAC) is considered a reliable surrogate marker for survival of TNBC [4]. Approximately, 30 % of the pCR rate was reported to be achieved by NAC administering both anthracyclines and taxanes in TNBCs, and achieving a pCR associated with a good prognosis [4]. In contrast, TNBC with a non-pCR was reported to display a markedly worse prognosis and are required to undergo treatment with new investigational therapeutic agents [4, 5]. Therefore, the reliable prediction of pCR should be made as early as possible before drugs with unknown efficacies are administered. Many studies have focused on predicting pCR from biopsy specimens but there have been no reliable markers associated with pCR in TNBC with the exception of the Ki-67 [6, 7]. Gene expression analyses have been proposed useful for predicting pCR [8, 9] but these techniques are not necessarily applicable in clinical settings, and sufficient validation has not been performed. Therefore, the development of the ability to predict pCR from biopsy specimens has remained a major challenge in clinical research.

Preclinical studies were recently reported that cytotoxic agents could partially exert their antitumor activities by inducing an immune response against tumor cells [10, 11]. The demise of immunogenic cells induced by cytotoxic agents allows cross-presentation of antigens and induction of tumor-specific cytotoxic T cells. Cytotoxic T cells (CD8+ T cells) have been reported to be associated with a higher pCR rate following NAC and a better outcome in the patients with breast cancer [12–14]. In addition, the regulatory T cells defined as forkhead box protein 3 (FOXP3)+ T cells have a critical role in suppressing antitumor immunity [15, 16]. However, it is also true that the prognostic and predictive roles of FOXP3 have remained in dispute; breast cancer tumors infiltrated with FOXP3+ T cells were reported to be less sensitive to cytotoxic chemotherapy and have a worse prognosis by some investigators [12, 17] but others indicated that breast tumors with FOXP3+ T cells achieved a higher pCR rate after NAC and had a better prognosis [18, 19]. These discrepancies could be due to the differences in the studied breast cancer subtypes and therapeutic regimens. The density of CD8+ and FOXP3+ T cells has previously been demonstrated to depend on the breast cancer subtype [12]. In addition, the therapeutic regimens and sensitivity to drugs could enormously vary across breast cancer subtypes. Therefore, the predictive roles of CD8 and FOXP3 should be investigated in a relatively homogeneous patient cohorts, namely in only one subtype and in those treated with current standard regimen for the subtype.

As in the immune response by T cells, neoangiogenesis has been considered important in breast cancer [20–22]. Neoangiogenesis is frequently co-regulated with tumor-infiltrating lymphocytes and increased neoangiogenesis in responders to neoadjuvant aromatase inhibitor, as reported by increase in the vasohibin-1 positive ratio (VPR) derived from the CD31 to vasohibin-1 ratio [23]. Therefore, the evaluation of neoangiogenesis combined with CD8+ and FOXP3+ infiltration is required for assessing the prediction of pCR.

Here we studied the potential roles of CD8 and FOXP3 via immunohistochemical double-staining in predicting pCR of the patients, together with analyses of neoangiogenesis, basal and proliferation markers, in a relatively larger TNBC cohort than previous studies and in a cohort treated with the current standard regimen of NAC.

Materials and methods

Patients and sample selection

In this retrospective study, 110 unselected TNBC patients who received NAC at three Japanese institutions (Tohoku University Hospital, Sendai, Japan; Tohoku Kosai Hospital, Sendai, Japan; and Nahanishi Clinic, Okinawa, Japan) between 2009 and 2012 were consecutively included. All biopsy specimens prior to NAC were fixed in 10 % neutral-buffered formalin and embedded in paraffin. The three institutional review boards approved the protocol of this study, which was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients.

Immunohistochemical double-staining and quantification of CD8 and FOXP3

The formalin-fixed paraffin-embedded specimens were cut into 4 μm thick sections for immunohistochemistry. Briefly, the antigen retrieval of FOXP3 was performed by autoclaving, and the anti-FOXP3 antibody reaction (clone: 236A/E7, Abcam) was performed. Next, CD8 antigen retrieval was performed and the anti-CD8 antibody reaction (clone: C8/144B, Nichirei) was performed. After the procedure for the biotin-streptavidin reaction, Vector Blue® was used to visualize the binding of the anti-CD8 antibody (blue), in contrast to FOXP3 (brown) [24].

To quantify the infiltration of CD8+ or FOXP3+ T cells, four non-overlapping fields with high numbers of tumor-infiltrating lymphocytes on hematoxylin–eosin-stained glass slides were selected. In the same fields of double-staining with CD8 and FOXP3 as the above four fields, the number of CD8+ or FOXP3+ lymphocytes was counted under high power magnification (×400). The mean number of CD8+ or FOXP3+ lymphocytes per field was