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Estrogen-related receptor γ modulates cell proliferation and estrogen signaling in breast cancer

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ABSTRACT

Breast cancer is primarily a hormone-dependent tumor that can be regulated by status of steroid hormones including estrogen and progesterone. Estrogen-related receptors (ERRs) are orphan nuclear receptors most closely related to estrogen receptor (ER) and much attention has been recently paid to the functions of ERRs in breast cancer in terms of the interactions with ER. In the present study, we investigated the expression of ERRγ in human invasive breast cancers by immunohistochemical analysis ($n = 110$) obtained by radical mastectomy. Nuclear immunoreactivity of ERRγ was detected in 87 cases (79%) and tended to correlate with the lymph node status. No significant associations were observed with other clinicopathological characteristics, including the expression levels of both estrogen and progesterone receptors. In MCF-7 breast cancer cells, we demonstrated that ERRγ mRNA was up-regulated dose-dependently by estrogen, and that this up-regulation of ERRγ mRNA by estrogen was abolished by ICI 182,780 treatment. We also demonstrated that exogenously transfected ERRγ increased MCF-7 cell proliferation. Furthermore, ERRγ enhanced estrogen response element (ERE)-driven transcription in MCF-7 cells. In 293T cells, ERRγ could also stimulate ERE-mediated transcription with or without ERα. These results suggest that ERRγ plays an important role as a modulator of estrogen signaling in breast cancer cells.

1. Introduction

Estrogen-signaling pathways are involved in the growth and development of breast tumors through the activation of estrogen receptor α (ERα) [1]. The cells of most breast cancers express high levels of ERα and exhibit estrogen-dependent proliferation. ERs are the members of the nuclear receptor superfamily and regulate various cellular events, including cell growth and apoptosis, by acting as transcription factors activating the expression of target genes. Therefore, comprehensive understanding of estrogen-signaling pathways in breast cancer is required for both treatment and diagnosis of the disease.

Recently, several researchers have focused on estrogen-related receptors (ERRs) as modulating factors for estrogen-signaling pathways [2,3]. ERRs (ERRα, ERRβ, and ERRγ) are orphan nuclear receptors that possess certain homologies to ER but cannot bind estrogen. In ERR-mediated transcriptional activation, coactivators are required in the interaction between the receptors and basal transcriptional machinery. Among such coactivators, peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α) and PGC-1β have been revealed to play important roles in ERR-mediated transcription [4–6]. In addition to PGC-1α/β, other coactivators have been shown to associate with both ERRs and ERs, suggesting that transcriptional cofactors are partially shared between ERs and ERRs. Furthermore, ERRs can bind to estrogen response elements (EREs) as well as ERR response elements (ERREs), suggesting that ERRs can affect ER-mediated signaling.

In vivo functions of ERRα and ERRγ were partly revealed using knockout mice. ERRα-knockout mice are viable but exhibit a phenotype characterized by reduced body weight, peripheral fat deposits, and resistance to high-fat diet-induced obesity [7]. On the

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other hand, ERRγ-null mice die during the early postnatal period as a result of abnormal heart function; these mice fail to make the transition at birth from the utilization of glucose as a fetal energy substrate to mitochondrial fatty-acid oxidation (FAO) [8]. The expression of ERRα is more abundant than that of the other 2 ERR subtypes and is detected in tissues with high metabolism, such as the heart, kidney, intestinal tract, skeletal muscle, and brown adipose tissue [9]. The expression patterns of ERRβ and ERRγ are more restricted, but these receptors are abundantly expressed in the heart and kidneys [9,10]. We have previously shown that ERRα and ERRγ are upregulated in preadipocyte cells and pluripotent mesenchymal cells under adipogenic conditions and that they positively regulate lipid accumulation in preadipocyte cells [11,12].

On the other hand, ERR is thought to be involved in the development of human cancer [13,14]. Expressions of ERRα and ERRγ mRNAs are associated with an unfavorable and favorable prognosis of breast cancer, respectively [15]. Expression of ERRα protein in breast tumors correlates with an increased risk of recurrence and a poor prognosis [13]. In human prostate cancer, patients with high ERRα and low ERRγ immunoreactivities show poor cancer-specific survival [16]. However, there has been no study investigating the association of ERRγ protein expression with breast cancer, and its role is yet to be elucidated.

In the present study, we evaluated the expression of ERRγ in human breast cancers by using immunohistochemistry; we then investigated the correlation between the ERRγ expression levels and clinicopathophysiological findings. Furthermore, we showed the estrogen-induced expression of ERRγ in human breast cancer MCF-7 cells and the stimulating effects of ERRγ on proliferation of MCF-7 cells. Finally, we revealed that ERRγ elevates ER-mediated transcription.

2. Materials and methods

2.1. Tissue selections and patient characteristics

Between January 2005 and March 2006, 110 consecutive patients were diagnosed with invasive breast cancer using a vacuum-assisted biopsy device (Mammotome®, Ethicon Endo-surgery, Inc., Cincinnati, OH) at Saitama Medical University Hospital. Formalin-fixed, paraffin-embedded sections obtained by biopsy or surgery were used in this study. The study was approved by the institutional review board at Saitama Medical University, and informed consent was obtained from all patients. Patient age ranged from 32 to 89 years (mean, 59.5). The clinicopathological characteristics of the series are presented in Table 1.

2.2. Antibodies

Anti-Flag M2 and anti-β-actin antibodies were purchased from Sigma–Aldrich (St. Louis, MO). Anti-ERRγ antibody was generated from rabbit serum using a glutathione S-transferase (GST) fusion protein with amino acids 2–51 of human ERRγ protein as an antigen. The antiserum was then purified using an affinity column filled with GST protein-coupled Affi-Gel 10 (Bio-Rad, Hercules, CA) to remove anti-GST antibody. The characterization of the antibody was previously confirmed by western blot analysis in pcDNA3-Flag-hERRγ-transfected 293T cells [16].

2.3. Immunohistochemistry

Immunohistochemical analysis for ERRγ was performed using an EnVision+ visualization kit (Dako, Carpinteria, CA), as previously described [16]. Tissue sections (6 μm) were deparaffinized, rehydrated through graded ethanol, and rinsed in Tris-buffered saline with 0.05% Tween-20 (TBST). To retrieve the antigens, the sections were heated in an autoclave at 121 °C for 10 min in 10 mM sodium citrate buffer (pH 6.0). The sections were blocked with endogenous peroxidase using 0.3% H_2O_2 and incubated in 10% bovine serum for 30 min. The primary antibody, a polyclonal antibody for ERRγ (1:1000 dilution), was applied and incubated at 4 °C overnight. The sections were rinsed in TBST and incubated with EnVision+ and anti-rabbit antibody for 1 h at room temperature. The antigen-antibody complex was visualized with 3, 3′-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris–HCl buffer [pH 7.6], and 0.006% H_2O_2). As a positive control, a section of human kidney tissue was immunostained with the anti-ERRγ antibody in the same manner as described above. Rabbit IgG was used in place of the primary antibody as a negative control.

Table 1
Relationship between immunoreactivity of ERRγ and clinicopathological findings in invasive breast cancer (n = 110).

Clinical findings	Immunoreactive score of ERRγ[a]		P value
	High (n = 87)	Low (n = 23)	
Age	59.0 ± 14.7	59.8 ± 14.7	0.83
≤50	24	7	0.99
>50	63	16	
Menopause			
Pre	25	6	0.99
Post	62	17	
pT			
≤20 mm	41	13	0.61
>20 mm	29	6	
Stage			
I, II	81	22	0.97
III, IV	6	1	
Grade			
I	32	8	0.95
II, III	32	9	
ER			
Positive (PS ≥ 3)	57	13	0.43
Negative (PS ≤ 2)	30	10	
PgR			
Positive (PS ≥ 3)	35	8	0.78
Negative (PS ≤ 2)	51	15	
HER2			
Positive	19	7	0.28
Negative	58	10	
Lymph node			
Positive (n ≥ 4)	12	0	0.06
Negative (n ≤ 3)	51	16	

ER, estrogen receptor; ERR, estrogen-related receptor; PgR, progesterone receptor; TS, total score; PS, proportion score.
[a] ERRγ immunoreactive scores of 0–3 and 4–8 were defined as low and high immunoreactivity, respectively.

2.4. Immunohistochemical assessment

Slides were evaluated for the proportion (proportion score [PS]: (0) none; (1) <1/100; (2) 1/100-1/10; (3) 1/10-1/3; (4) 1/3-2/3; and (5) >2/3) and staining intensity (intensity score [IS]: (0) none; (1) weak; (2) moderate; and (3) strong) of positively stained cells. The total immunoreactivity score (TS: 0, 2–8) was determined as the sum of the proportion and intensity scores [17]. Two investigators (H.T. and A.O.) evaluated the tissue sections independently. If the immunoreactivity score differed between the 2 investigators, a third investigator (T.S.) evaluated the tissue sections, and the average immunoreactivity score was used. When the 2 investigators found it difficult to evaluate the TS of the heterogeneous cancerous lesions, the third investigator estimated the latter and decided the immunoreactivity score. We defined a TS of 3 as the cut-off for high ERRγ immunoreactivity to identify a potential correlation between ERRγ expression in the malignant epithelium and clinicopathological characteristics.

2.5. Statistical analyses

The correlation between the immunoreactivity score and clinicopathological characteristics was evaluated with the chi-square test. P values < 0.05 were regarded as statistically significant. Differences between the 2 groups in luciferase and cell proliferation assays were analyzed using a 2-sample, 2-tailed Student's t test. A P value < 0.05 was considered to be significant. All data are presented in the text and figures as the mean (standard deviation (SD)).

2.6. Plasmid construction

Human ERα (hERα, amino acids 2-595) and ERRγ (hERRγ, amino acids 2-458) were N-terminally tagged with Flag and subcloned into pcDNA3 vector (pcDNA3-Flag-hERα and pcDNA3-Flag-hERRγ, respectively).

2.7. Cell culture and transfection

The 293T and MCF-7 cells were purchased from American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2. 17β-estradiol and ICI 182,780 were purchased from Sigma and Tocris Bioscience, respectively. Transfection of hERRγ was performed using 2 μg of pcDNA3-Flag-hERRγ and Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. After 48 h, cell extracts were analyzed by western blot analysis.

2.8. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA extraction, first-strand cDNA synthesis, quantitative PCR, and primer sequences have been described elsewhere [11,18,19]. Fold induction of mRNA expression levels was determined by comparing the mRNA levels of the estrogen-treated samples with those of the vehicle-treated control.

2.9. Luciferase assay

MCF-7 and 293T cells were plated in 24-well culture plates at a density of 10,000 cells/well in phenol red-free medium containing 5% charcoal-stripped serum and transfected with 0.1 μg of ERE-tk-luc [20], together with 0.02 μg of pRL-cytomegalovirus (CMV; Promega, Madison, WI) using a Lipofectamine 2000 reagent (Invitrogen). Twelve hours after transfection, cells were treated with 100 nM 17β-estradiol or vehicle (ethanol) for 24 h, and luciferase activities were determined by a MicroLumatPlus microplate luminometer (Berthold Technologies, Bad Wildbad, Germany) using a Dual-Luciferase Assay System (Promega). Data are expressed as mean (SD) of 3 independent experiments performed in triplicate.

2.10. Cell proliferation assay

MCF-7 cells were seeded in 96-well plates at a density of 5000 cells/well in phenol red-free medium containing 5% charcoal-stripped serum for 24 h. Then, pcDNA3-Flag or pcDNA3-Flag-hERRγ was transfected for 12 h and incubated with 100 nM estradiol or vehicle for 72 h. Cell proliferation was examined by a tetrazolium salt (WST-8) assay kit (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocol.

3. Results

3.1. Correlation of ERRγ protein expression with clinicopathological values of invasive breast cancer

To investigate the expression levels of ERRγ protein in breast cancer, immunohistochemical analysis was performed using 110 invasive breast cancers (Fig. 1). Strong nuclear immunoreactivity of ERRγ was detected in 79% of breast cancer specimens (Fig. 1A and C). A human kidney tissue was immunostained with the ERRγ antibody as a positive control, and ERRγ immunoreactivity was also observed in the nuclei of kidney tubule cells (Fig. 1E). Statistical analysis showed that the nuclear immunoreactivity of ERRγ tended to correlate with lymph node status ($P = 0.06$) while no significant associations were found with other clinicopathological characteristics (Table 1). In the DCIS component of the ERRγ-positive invasive carcinomas, ERRγ immunoreactivity was detected in the nucleus. ERRγ immunoreactivity was also detected in the nuclei of normal mammary epithelium and intratumoral stromal cells (data not shown).

3.2. Upregulation of ERRγ in estrogen-treated MCF-7 cells

Next, we examined the expressional regulation of 3 ERR genes (ERRα, ERRβ, and ERRγ) by estrogen in an ERα-positive breast cancer cell line (MCF-7) using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) (Fig. 2A–C). ERRγ mRNA level was significantly up-regulated by 6-fold at 3 h after estrogen stimulation. ERRα mRNA was also slightly up-regulated by 2.5-fold in a time-dependent manner while ERRβ mRNA was not largely influenced by estrogen. In addition, ERRγ mRNA was up-regulated dose-dependently by estrogen (Fig. 2D). This estrogen-dependent up-regulation of ERRγ mRNA was abolished by ICI 182,780 treatment, while ICI 182,780 itself did not up-regulate ERRγ mRNA expression (Fig. 2D). These results suggested that ERRγ expression is regulated by estrogen.

3.3. ERRγ contributes to estrogen-dependent and estrogen-independent proliferation in breast cancer cells

To further assess the role of ERRγ in breast cancer, we performed a gain-of-function study for ERRγ. Under an estrogen-deprived culture condition, ERRγ-overexpressing MCF-7 cells exhibited a significantly higher growth rate compared with control cells expressing empty vector at days 3 and 4 (Fig. 3A). Furthermore, growth of ERRγ-overexpressing MCF-7 cells was also stimulated in the presence of 100 nM estrogen (Fig. 3B). We confirmed that the ERRγ protein was overexpressed in MCF-7 cells after transient transfection with ERRγ expression plasmid by immunoblotting (Fig. 3C). These results indicate that ERRγ promotes proliferation of breast cancer cells regardless of the presence or absence of estrogen.

3.4. ERRγ promotes ER-mediated transcription

To examine whether ERRγ influences ER-ERE-mediated transcription, a luciferase reporter vector containing an ERE (ERE-tk-luc) was introduced into 293T cells with or without ERRγ expression vector (Fig. 4A). The result showed that ERRγ significantly stimulated ER-ERE-mediated transactivation in 293T cells when the cells were transfected with ERα and treated with estrogen. We also observed that the estrogen-dependent transactivation was elevated depending on the increasing amount of ERRγ in MCF-7 cells (Fig. 4B). These results indicate that ERRγ stimulates ER transcription activity in response to estrogen. Next, we examined the effect of ERRγ on ERE-mediated transcription under the

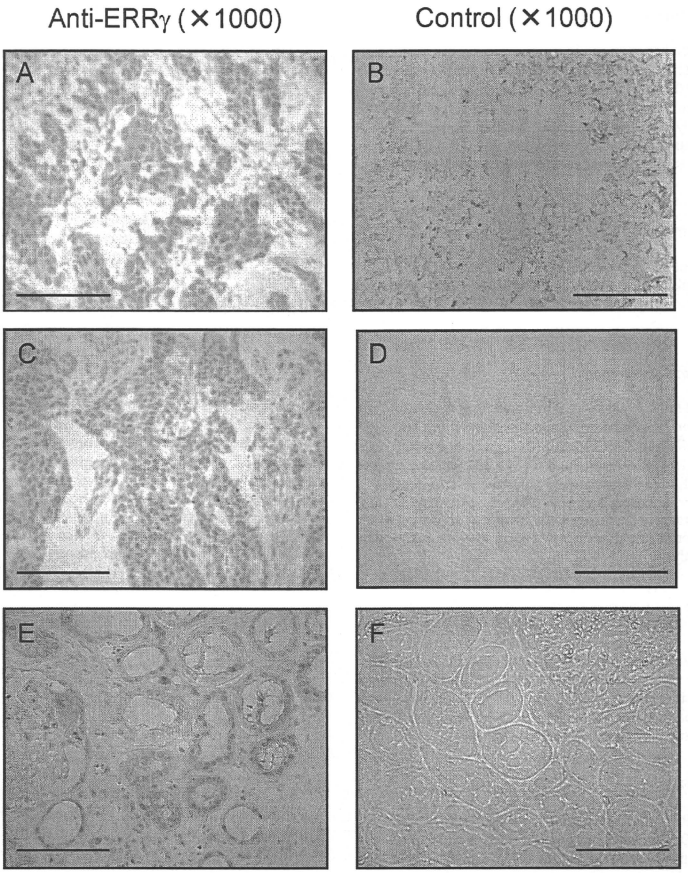

Fig. 1. Immunohistochemistry of estrogen-related receptor (ERR) γ in breast cancer. Representative immunohistochemical staining of breast cancer tissues (A–D) and normal kidney tissue (E and F) with anti-ERRγ (A, C, and E) and rabbit IgG (B, D, and F). Positive staining for ERRγ was observed in the nuclei of breast cancer cells, as well as of kidney tubule cells. Bar, 100 μm.

estrogen-free condition. Intriguingly, the transcriptional activity of the ERE-luciferase reporter construct was dose-dependently stimulated by ERRγ in an estrogen-independent manner (Fig. 4C).

4. Discussion

In the present study, ERRγ immunoreactivity was detected in 79% of invasive breast cancers ($n = 110$) by immunohistochemistry and tended to correlate with the lymph node status ($P = 0.06$). Generally the ER-positive breast cancer has a better prognosis than ER-negative one. Consistently with this observation, immunostainings of some estrogen responsive genes, such as progesterone receptor [21], were known to be correlated with a good prognosis. On the other hand, immunostainings of some estrogen responsive genes, such as cathepsin D [22], ERRα [13,23] and Efp [24], were shown to be as poor prognostic factors. Indeed, the immunoreactivities of Efp and ERRα in breast cancer specimens were reported to be positively correlated with lymph node status. Besides, as demonstrated in the present study, ERRγ could function as an estrogen responsive gene and facilitate the proliferation of MCF-7 cells. Notably, ERRγ could also stimulate the ERE-mediated transcription by itself. These findings may explain that the immunoreactivity of ERRγ tends to be correlated with the lymph node status in breast cancer. In our immunohistochemical analysis of invasive breast carcinomas, no significant association between ERRγ and ERα expressions was observed. Although the present study is the first to evaluate the expression of ERRγ protein in human breast cancers by using immunohistochemistry, a previous association study reported that overexpression of ERRγ mRNA is associated with ER-positive and PgR-positive status in primary breast tumors

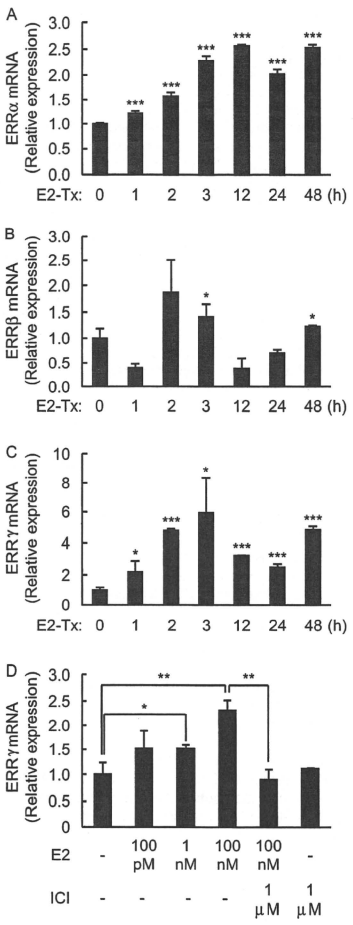

Fig. 2. Regulation of ERRs in MCF-7 cells by estrogen stimulation. (A–C) MCF-7 cells were treated with 100 nM 17β-estradiol for 48 h. ERRα (A), ERRβ (B), and ERRγ (C) mRNA levels were examined at indicated time points by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR), and the results are shown as fold change over the expression level at 0 h. $^{*}P<0.05$ compared with 0 h; $^{***}P<0.001$ compared with 0 h (by Student's *t* test). (D) MCF-7 cells were treated with 17β-estradiol (100 pM, 1 nM, or 100 nM) and/or ICI 182,780 (1 μM), or vehicle for 2 h. ERRγ mRNA levels were examined by qRT-PCR, and the results are shown as fold change over the expression level with vehicle treatment. $^{*}P<0.05$ compared with vehicle treatment: $^{**}P<0.01$ compared with vehicle treatment or 100 nM E2 treatment (by Student's *t* test).

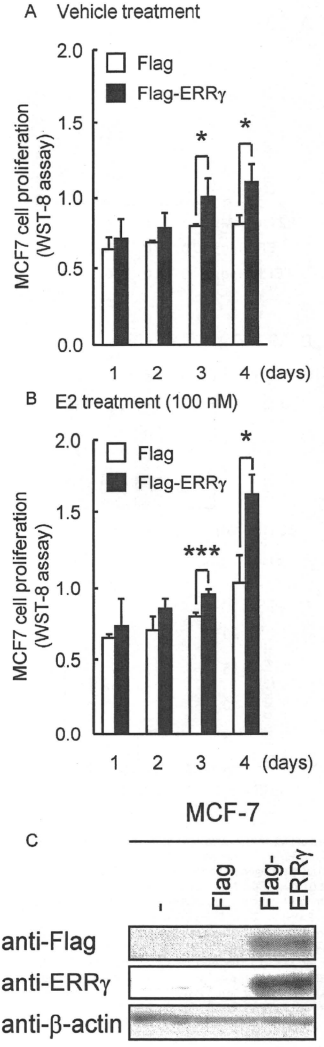

Fig. 3. ERRγ overexpression promotes estrogen-dependent and estrogen-independent proliferation of MCF-7 cells. (A and B) MCF-7 cells were transfected with pcDNA3-Flag-hERRγ for 24 h and then treated with vehicle (A) or 100 nM 17β-estradiol (E2) (B) for 4 days. Cell proliferation was examined using a tetrazolium salt (WST-8) assay kit. $^{*}P<0.05$ compared with vehicle; $^{***}P<0.001$ compared with vehicle (by Student's *t* test). (C) Total cell lysates from the parental MCF-7 cells (–) or the MCF-7 cells transfected with pcDNA3-Flag (Flag) or pcDNA3-Flag-hERRγ (Flag-ERRγ) for 48 h were immunoblotted by anti-Flag, -ERR, or -β-actin antibodies.

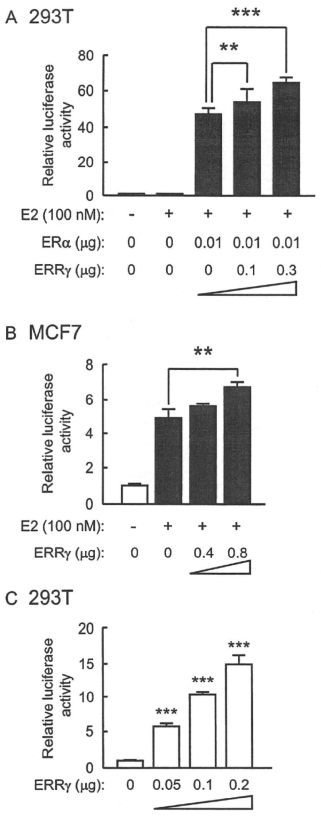

Fig. 4. Effect of ERγ overexpression on ERE-mediated transcription activity in 293T (A and C) and MCF-7 (B) cells. (A) The 293T cells were transfected with a DNA mixture of 100 ng of estrogen response element (ERE)-tk-Luc, 0.02 μg of pRL-CMV with or without 0.01 μg of pcDNA3-Flag-ERα, and increasing amounts of pcDNA3-Flag-hERRγ. After a 12-h incubation, cells were treated with 17β-estradiol (100 nM, E2) or vehicle (EtOH) for 24 h. (B) MCF-7 cells were transfected with a DNA mixture of 100 ng of ERE-tk-Luc, 0.02 μg of pRL-CMV, and increasing amounts of pcDNA3-Flag-hERRγ. After a 12-h incubation, cells were treated with 17β-estradiol (100 nM) or vehicle for 24 h. (C) The 293T cells were transfected with a DNA mixture of 100 ng of ERE-tk-Luc, 0.02 μg of pRL-CMV, and increasing amounts of pcDNA3-Flag-hERRγ, and incubated for 36 h. $^{**}P<0.01$; $^{***}P<0.001$ compared with corresponding conditions with no ERRγ transfection (by Student's *t* test).

($n=38$) [15]. One possible explanation for this discrepancy is that the expression level of ERRγ protein did not necessarily correlated with that of ERRγ mRNA in breast cancer cells. Alternatively, this may have resulted from the differences in numbers or types of breast tumor specimens. One third of the clinical cases of ER-positive/PR-positive breast tumor patients treated with tamoxifen do not respond to initial treatment, and the remaining 70% are still at risk for relapse in the future. Riggins et al. recently reported that ERRγ expression was increased in a tamoxifen-resistant invasive lobular carcinoma (ILC) cell model [25]. In line with this notion, we observed that the ERRγ mRNA level in ER-negative breast cancer MDA-MB-231 cells was higher than that in MCF-7 cells (data not shown), suggesting that ERRγ could be involved in tamoxifen resistance of breast cancer cells.

In the present study, we demonstrated that ERRγ expression is stimulated dose-dependently by estrogen in MCF-7 breast cancer cells, while this stimulation was abolished by ICI 182,780 (Fig. 2C and D). Supporting our results, several estrogen receptor-binding sites (ERBSs) have been found within the second intron of the human ERRγ gene by genome-wide chromatin immunoprecipitation (ChIP)-on-chip analysis using MCF-7 cells [26]. Thus, it is possible that ERRγ expression is regulated by ER through these ERBSs. In addition, we found that ERRγ overexpression promotes the growth of MCF-7 cells. Moreover, a transcriptional reporter assay revealed that ERRγ enhances ER-mediated transcription in MCF-7 cells. ERR recognizes not only the consensus sequence TCAAGGTA, referred to as ERRE, but also the ERE that is bound to ER [10]. Thus, ERR control of the transcription of target genes partly overlaps with ERα [2,10,27]. It has also been reported that ERR associates with ER and modulates ER-mediated transcription [28,29]. Besides, ERRγ itself could stimulate the ERE-mediated transcription in ER-negative 293T cells in an estrogen-independent manner. These findings together with our data suggest that ERRγ, a downstream target of ERα itself, could stimulate the growth of breast cancer cells by modulating estrogen-signaling pathways or transcriptional activity of ERα.

Our data and a previous report indicate that, as in the case of ERRγ mRNA, expression of ERRα mRNA is induced by estrogen in MCF-7 cells [23]. ERRα immunoreactivity was also noted to be significantly associated with an increased risk of recurrence and adverse clinical outcome in breast cancer, but it does not correlate with ERα immunoreactivity [13]. Although ERR possesses characteristics that are structurally and functionally similar to ER, ERR has no natural ligand and regulates expression of target genes that are distinct from those of ER, except for coregulated genes. Therefore, ERR is assumed to have ER-independent functions in breast cancer. For example, ERRs are implicated in the transcriptional response to hypoxia and the growth of solid tumors. The development of intratumoral hypoxia is a universal hallmark of rapidly growing solid tumors, and the adaptive response to hypoxia is mediated primarily through the hypoxia-inducible factor (HIF)-dependent transcriptional program. HIF regulates gene networks involved in glucose uptake and metabolism in tumor angiogenesis. ERRγ, as well as ERRα and ERRβ, can physically interact with HIF1α, HIF2α, and HIF1β both *in vitro* and *in vivo* to enhance HIF-mediated gene transcription, suggesting that ERRs may be required for HIF function [30].

In summary, our results suggest that ERRγ expression is induced by estrogen in breast cancer cells, and expression of this receptor promotes cancer cell proliferation by enhancing ERE-mediated transcription. These results further suggest that pharmacological modulation of ERRγ activity may be clinically useful to prevent and/or treat breast cancer.

Conflict of interest

The authors declare that there are no conflicts of interest.

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References

[1] N. Platet, A.M. Cathiard, M. Gleizes, M. Garcia, Estrogens and their receptors in breast cancer progression: a dual role in cancer proliferation and invasion, Crit. Rev. Oncol. Hematol. 51 (1) (2004) 55–67.

[2] V. Giguere, To ERR in the estrogen pathway, Trends Endocrinol. Metab. 13 (5) (2002) 220–225.

[3] N. Yang, H. Shigeta, H. Shi, C.T. Teng, Estrogen-related receptor, hERR1, modulates estrogen receptor-mediated response of human lactoferrin gene promoter, J. Bio. Chem. 271 (10) (1996) 5795–5804.

[4] D. Knutti, A. Kralli, PGC-1, a versatile coactivator, Trends Endocrinol. Metab. 12 (8) (2001) 360–365.

[5] P. Puigserver, B.M. Spiegelman, Peroxisome proliferator-activated receptor-g coactivator 1a (PGC-1a): transcriptional coactivator and metabolic regulator, Endocr. Rev. 24 (1) (2003) 78–90.

[6] J. Lin, P.T. Tarr, R. Yang, J. Rhee, P. Puigserver, C.B. Newgard, B.M. Spiegelman, PGC-1b in the regulation of hepatic glucose and energy metabolism, J. Biol. Chem. 278 (33) (2003) 30843–30848.

[7] J. Luo, R. Sladek, J. Carrier, J.A. Bader, D. Richard, V. Giguère, Reduced fat mass in mice lacking orphan nuclear receptor estrogen-related receptor α, Mol. Cell. Biol. 23 (22) (2003) 7947–7956.

[8] W.A. Alaynick, R.P. Kondo, W. Xie, W. He, C.R. Dufour, M. Downes, J.W. Jonker, W. Giles, R.K. Naviaux, V. Giguère, R.M. Evans, ERRg directs and maintains the transition to oxidative metabolism in the postnatal heart, Cell Metab. 6 (1) (2007) 13–24.

[9] A.L. Bookout, Y. Jeong, M. Downes, R.T. Yu, R.M. Evans, D.J. Mangelsdorf, Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network, Cell 126 (4) (2006) 789–799.

[10] V. Giguere, Transcriptional control of energy homeostasis by the estrogen related receptors, Endocr. Rev. 29 (6) (2008) 677–696.

[11] N. Ijichi, K. Ikeda, K. Horie-Inoue, K. Yagi, Y. Okazaki, S. Inoue, Estrogen-related receptor a modulates the expression of adipogenesis-related genes during adipocyte differentiation, Biochem. Biophys. Res. Commun. 358 (3) (2007) 813–818.

[12] M. Kubo, N. Ijichi, K. Ikeda, K. Horie-Inoue, S. Takeda, S. Inoue, Modulation of adipogenesis-related gene expression by estrogen-related receptor g during adipocytic differentiation, Biochim. Biophys. Acta 1789 (2) (2009) 71–77.

[13] T. Suzuki, Y. Miki, T. Moriya, N. Shimada, T. Ishida, H. Hirakawa, N. Ohuchi, H. Sasano, Estrogen-related receptor a in human breast carcinoma as a potent prognostic factor, Cancer Res. 64 (13) (2004) 4670–4676.

[14] R.J. Kraus, E.A. Ariazi, M.L. Farrell, J.E. Mertz, Estrogen-related receptor a1 actively antagonizes estrogen receptor-regulated transcription in MCF-7 mammary cells, J. Bio. Chem. 277 (27) (2002) 24826–24834.

[15] E.A. Ariazi, G.M. Clark, J.E. Mertz, Estrogen-related receptor a and estrogen related receptor g associated with unfavorable and favorable biomarkers, respectively, in human breast cancer, Cancer Res. 62 (22) (2002) 6510–6518.

[16] T. Fujimura, S. Takahashi, T. Urano, N. Ijichi, K. Ikeda, J. Kumagai, T. Murata, K. Takayama, K. Horie-Inoue, Y. Ouchi, M. Muramatsu, Y. Homma, S. Inoue, Differential expression of estrogen-related receptors b and g (ERRb and ERRg) and their clinical significance in human prostate cancer, Cancer Sci. 101 (3) (2010) 646–651.

[17] D.C. Allred, G.M. Clark, R. Elledge, S.A. Fuqua, R.W. Brown, G.C. Chamness, C.K. Osborne, W.L. McGuire, Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer, J. Natl. Cancer Inst. 85 (3) (1993) 200–206.

[18] K. Horie-Inoue, K. Takayama, H.U. Bono, Y. Ouchi, Y. Okazaki, S. Inoue, Identification of novel steroid target genes through the combination of bioinformatics and functional analysis of hormone response elements, Biochem. Biophys. Res. Commun. 339 (1) (2006) 99–106.

[19] K. Takayama, K. Kaneshiro, S. Tsutsumi, K. Horie-Inoue, K. Ikeda, T. Urano, N. Ijichi, Y. Ouchi, K. Shirahige, H. Aburatani, S. Inoue, Identification of novel androgen response genes in prostate cancer cells by coupling chromatin immunoprecipitation and genomic microarray analysis, Oncogene 26 (30) (2007) 4453–4463.

[20] K. Ikeda, S. Ogawa, T. Tsukui, K. Horie-Inoue, Y. Ouchi, S. Kato, M. Muramatsu, S. Inoue, Protein phosphatase 5 is a negative regulator of estrogen receptor-mediated transcription, Mol. Endocrinol. 18 (5) (2004) 1131–1143.

[21] B.H. Mason, I.M. Holdaway, P.R. Mullins, L.H. Yee, R.G. Kay, Progesterone and estrogen receptors as prognostic variables in breast cancer, Cancer Res. 43 (6) (1983) 2985–2990.

[22] A.K. Tandon, G.M. Clark, G.C. Chamness, J.M. Chirgwin, W.L. McGuire, Cathepsin D and prognosis in breast cancer, N. Engl. J. Med. 322 (5) (1990) 297–302.

[23] D. Liu, Z. Zhang, W. Gladwell, C.T. Teng, Estrogen stimulates estrogen-related receptor a gene expression through conserved hormone response elements, Endocrinology 144 (11) (2003) 4894–4904.

[24] T. Suzuki, T. Urano, T. Tsukui, K. Horie-Inoue, T. Moriya, T. Ishida, M. Muramatsu, Y. Ouchi, H. Sasano, S. Inoue, Estrogen-responsive finger protein as a new potential biomarker for breast cancer, Clin. Cancer Res. 11 (17) (2005) 6148–6154.

[25] R.B. Riggins, J.P. Lan, Y. Zhu, U. Klimach, A. Zwart, L.R. Cavalli, B.R. Haddad, L. Chen, T. Gong, J. Xuan, S.P. Ethier, R. Clarke, ERRg mediates tamoxifen resistance in novel models of invasive lobular breast cancer, Cancer Res. 68 (21) (2008) 8908–8917.

[26] J.S. Carroll, C.A. Meyer, J. Song, W. Li, T.R. Geistlinger, J. Eeckhoute, A.S. Brodsky, E.K. Keeton, K.C. Fertuck, G.F. Hall, Q. Wang, S. Bekiranov, V. Sementchenko, E.A. Fox, P.A. Silver, T.R. Gingeras, X.S. Liu, M. Brown, Genome-wide analysis of estrogen receptor binding sites, Nat. Genet. 38 (11) (2006) 1289–1297.

[27] G. Deblois, J.A. Hall, M.C. Perry, J. Laganière, M. Ghahremani, M. Park, M. Hallett, V. Giguère, Genome-wide identification of direct target genes implicates estrogen-related receptor a as a determinant of breast cancer heterogeneity, Cancer Res. 69 (15) (2009) 6149–6157.

[28] N. Yang, H. Shigeta, H. Shi, C.T. Teng, Estrogen-related receptor, hERR1, modulates estrogen receptor-mediated response of human lactoferrin gene promoter, J. Biol. Chem. 271 (10) (1996) 5795–5804.

[29] V. Bombail, F. Collins, P. Brown, P.T. Saunders, Modulation of ERa transcriptional activity by the orphan nuclear receptor ERRb and evidence for differential effects of long- and short-form splice variants, Mol. Cell. Endocrinol. 314 (1) (2010) 53–61.

[30] A. Ao, H. Wang, S. Kamarajugadda, J. Lu, Involvement of estrogen-related receptors in transcriptional response to hypoxia and growth of solid tumors, Proc. Natl. Acad. Sci. U.S.A. 105 (22) (2008) 7821–7826.

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Prognostic Factors in Young Japanese Women with Breast Cancer: Prognostic Value of Age at Diagnosis

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Objective: The primary objective of this study was to verify whether breast cancer patients aged <35 at diagnosis have poorer prognoses than those aged 35–39, in other words, to identify the prognostic value of age in younger premenopausal patients under 40 years old. The secondary objective was to assess prognostic factors specific for younger premenopausal patients.

Methods: We identified 242 consecutive patients who were diagnosed with stage I–III breast cancer before the age of 40 and underwent surgery between 1990 and 2004. We compared disease-free survival and overall survival in patients aged <35 years and those aged 35–39 years, and evaluated clinicopathological factors associated with disease-free survival or overall survival in each age group and in all patients under the age of 40.

Results: Ninety-nine (41%) patients were younger than 35 years and 143 (59%) were between 35 and 39 years. No significant difference in disease-free survival or overall survival was found between the two groups. In our cohort of patients under the age of 40, the independent factors associated with poor disease-free survival and overall survival included positive axillary lymph nodes and triple-negative status, but not age at diagnosis. Adverse prognostic factors also did not differ considerably between the two age groups.

Conclusions: Age at diagnosis was not an independent prognostic factor in our study. Our findings suggest that other clinicopathological features rather than age should be used to determine individualized treatment courses for breast cancer patients younger than 40 years.

Key words: breast cancer – young – disease-free survival – overall survival

INTRODUCTION

Many studies have reported that younger women with primary breast cancer have poorer prognoses than older women. The St Gallen international expert consensus reports from 1998 to 2007 concluded the age of <35 years was a high-risk factor for relapse in node-negative breast cancer patients and recommended adjuvant chemotherapy for most young women with breast cancer (1–5). However, the decision regarding chemotherapy in young patients must be made after taking into consideration not only the risk of relapse but also the age-specific problems caused by chemotherapy such as infertility, bone loss and changes in sexual function and appearance.

The cutoff value for classifying a patient as 'young' varies among studies and it is unclear whether the age of <35 years at diagnosis was an appropriate threshold to identify patients with primary breast cancer at high risk of relapse. It also remains to be determined whether Japanese patients aged <35 years at diagnosis have poorer prognoses since there have been few reports focusing on young Japanese women with breast cancer.

Prognostic factors in younger patients with primary breast cancer have been recently identified, but are not yet well

understood. A recent study showed that gene expression profile was a powerful predictor of disease outcome in young patients with breast cancer, but age was not an independent prognostic factor (6).

Gene expression profiling has identified intrinsic breast cancer subtypes that predict distinct clinical outcomes (7,8). In particular, triple-negative breast cancer, defined by the lack of expression of estrogen receptor (ER), progesterone receptor (PgR) and human epidermal growth factor receptor 2 (HER2), is known to be a subtype associated with poor clinical outcome. A high prevalence of triple-negative breast cancer has been reported to contribute to the poor prognosis of young African American women with breast cancer (9).

The primary objective of this study was to verify whether breast cancer patients aged <35 at diagnosis have poorer prognoses than those aged 35–39, in other words, to identify the prognostic value of age in younger premenopausal patients under 40 years old. The secondary objective was to assess the prognostic factors specific for younger premenopausal patients.

PATIENTS AND METHODS

Patients and Treatment

From the database of the National Cancer Center Hospital, Tokyo, Japan, we identified consecutive patients who were diagnosed with breast cancer before the age of 40 years and underwent surgery between January 1990 and December 2004. Only patients with stage I–III disease who underwent definitive surgery were included. Patients who had undergone preoperative adjuvant therapy or had excisional biopsy in a local clinic were also excluded because it is difficult to determine pathological factors influencing prognoses.

The complete medical records of patients enrolled in the study were reviewed. Information derived from the database and medical records included clinical and histological variables such as age; family history; pT (primary tumor) and pN (regional lymph node) status; histological type; histological grade; peritumoral vessel invasion (PVI) [including lymphatic vessel invasion (LVI) and blood vessel invasion (BVI)]; ER, PgR and HER2; tumor subtype stratified by hormone receptor (HR) and HER2 status; operative procedure; radiation therapy; adjuvant systemic therapy (chemotherapy and endocrine therapy).

Familial breast cancer (that does not fit hereditary breast cancer definition) was defined as breast cancer with a family history of one or more first- or second-degree relatives with breast cancer prior to or at the time of the patient's initial diagnosis (10,11). In all cases, pT and pN status were assessed according to the UICC TNM classification (6th edition) (12). Histological grade was evaluated according to Elston and Ellis (13). ER and PgR expression were determined by enzyme immunoassay or immunohistochemistry (IHC) (threshold for positivity: staining in more than 10% of tumor cells) (14). The definition of HER2 positive was a score 3+ by IHC (uniform, intense membrane staining in more than 10% of invasive cancer cells) and/or a 2.0 or higher of HER2/CEP17 (centromere probe chromosome 17) ratio by fluorescence *in situ* hybridization (15). On the basis of the expression profile of HR and HER2, all tumors were categorized into one of the four subtypes: HR+HER2−, HR+HER2+, HR−HER2+, HR−HER2− (triple-negative). HR-positive status (HR+) was defined as ER and/or PgR positivity, and HR-negative status (HR−) was defined as ER and PgR negativity. PVI was determined by the presence of tumor emboli within peritumoral endothelial-lined spaces and was assessed on hematoxylin and eosin-stained slides by making a distinction between lymphatic and blood vessels. LVI was graded as absent, focal to moderate (one to five foci of tumor thrombi in all the tumor specimens examined) or extensive (more than five foci of tumor thrombi in all the tumor specimens examined) (16). BVI was classified as either absent or present.

All patients received clinically necessary local treatment (breast-conserving surgery or mastectomy) in addition to sentinel node biopsy or complete axillary dissection. Postoperative breast irradiation was indicated for all patients who underwent breast-conserving surgery. After 1999, patients with pT3 presentation who had undergone mastectomy received postoperative radiation to the chest wall. Patients with four or more metastatic axillary lymph nodes received postoperative radiation to the axillary and supraclavicular regions. Adjuvant chemotherapy was followed by radiotherapy for all indicated patients. The adjuvant chemotherapy regimen widely used prior to 1993 comprised doxorubicin, cyclophosphamide (AC), methotrexate and 5-fluorouracil. After 1993, patients generally received four cycles of intravenous doxorubicin and AC. After 1999, high-risk patients received AC followed by taxane (docetaxel or paclitaxel). For women with endocrine-responsive disease aged <40 years, adjuvant endocrine therapy was indicated, such as tamoxifen for 2–5 years or the combination of tamoxifen for 5 years plus gonadotropin-releasing hormone analogues for at least 2 years. Patients who received adjuvant chemotherapy for endocrine-responsive disease were treated with tamoxifen immediately after the completion of chemotherapy.

Patients were followed up every 3–6 months during the first 5 years and every 6–12 months from 5 to 10 years. In addition to physical examination, annual mammography with or without breast ultrasound was performed for 10 years. Blood tests including two tumor markers (carcinoembryonic antigen and cancer antigen 15-3), chest X-ray, abdominal ultrasonography and bone scintigraphy were performed when the patients complained of any symptoms and/or tumor recurrence was suspected.

The study was conducted with support from the Health and Science Grants for Clinical Research in Cancer, as part of the investigations directed by the Ministry of Health, Labor and Welfare of Japan. The data on which the study was based were obtained in the course of daily clinical

practice and no additional burdens were imposed on patients. Hence, ethical approval was not required.

Statistical Methods

The χ^2 test (Pearson statistic) was used to determine the differences in clinical and pathological factors between two groups of patients. A P value of <0.01 was considered statistically significant.

The follow-up duration was calculated as the length of time between the date of diagnosis and the date of death or last contact. Disease-free survival (DFS) was defined as the time from surgical resection to the first of any of the following events: locoregional relapse, distant relapse, second primary breast cancer, any second (non-breast) malignancy or death from any cause. Locoregional relapse was defined as the reappearance of cancer in the ipsilateral breast, chest wall or regional lymph nodes. We classified distant relapse into two categories depending on metastatic sites: non-visceral (soft-tissue and/or bone) or visceral (including lung, liver, brain and other organs). Overall survival (OS) was defined as the time from surgical resection to death due to any cause, regardless of recurrence. DFS and OS curves were drawn by the Kaplan–Meier method and were compared among patient subsets using the log-rank test.

In univariate analyses, the following prognostic factors were evaluated for their potential associations with DFS and OS: age at the time of diagnosis, familial breast cancer, pT, pN, histological type, histological grade, LVI, BVI, tumor subtype stratified by HR and HER2 status, operative procedure, administration of radiation therapy and adjuvant systemic therapy. ER, PgR and HER2 were excluded from the prognostic analyses for DFS and OS because these factors are closely related to tumor subtype. Multivariate analysis of potential prognostic factors was performed to generate a Cox proportional hazards model. Multivariate models were created using age at diagnosis and other variables that showed significant association ($P < 0.01$) with DFS or OS on univariate analysis. All tests were two-tailed, with $P < 0.01$ being taken as an indicator of statistical significance. The statistical software SPSS version 12.0 (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses.

RESULTS

Patient Characteristics

Out of a total of 3944 patients who underwent surgery at the National Cancer Center Hospital, Tokyo, Japan, between January 1990 and December 2004, 242 patients were eligible for this study. Of which 99 (41.0%) were aged <35 years at diagnosis, and 143 (59.0%) were aged between 35 and 39 years (Table 1). The median age at diagnosis was 36 years (range 22–39 years). The distribution of various clinicopathological factors did not differ significantly between the two age groups. PgR positivity was observed in a higher percentage of patients aged 35–39 years than in those aged <35 years, but the proportion of patients falling into each of these four tumor subtypes did not differ significantly between the two groups. Sixty-nine percent of the 242 patients were classified as HR+HER2−, 10.3% were HR+HER2+, 5.8% were HR−HER2+ and 14.9% were HR−HER2− (triple-negative).

During a median follow-up of 80 months (range 5–186 months), 86 patients (35.5%) experienced DFS events [second primary breast cancer 3.7%; locoregional relapse 7.4%; distant relapse 24.4% (non-visceral 8.7%; visceral 15.7%)] and 51 patients (21.1%) died. No significant difference was found in DFS and OS between patients aged <35 years and those aged 35–39 years (Fig. 1). We did not also find a significant difference in frequency of occurrence of various DFS events between the two age groups (Table 1).

Univariate Analyses

For breast cancer patients under 40 years old, univariate analyses showed that significant adverse factors associated with both DFS and OS included higher T stage (pT3–4), positive lymph nodes (pN1–3), grade 3, extensive LVI, BVI, triple-negative status and adjuvant chemotherapy (Tables 2 and 3). With regard to adjuvant chemotherapy, patients who were treated with chemotherapy had significantly worse DFS and OS. No significant difference in survival was observed between the familial breast cancer group and the non-familial group.

Multivariate Analyses

For all patients under the age of 40, multivariate analyses identified positive axillary lymph nodes (pN1–pN3) and triple-negative status as independent factors associated with poor DFS and OS (Tables 2 and 3, and Fig. 2). Age, represented as either a categorical or a continuous variable, was not an independent prognostic factor in multivariate analyses. The independent factors negatively influencing DFS included pN1 (hazard ratio 3.69, 95% CI 1.61–8.47), pN2–pN3 (hazard ratio 6.55, 95% CI 2.72–15.75) and triple-negative status (hazard ratio 2.45, 95% CI 1.37–4.36). The independent adverse factors affecting OS included pN1 (hazard ratio 6.00, 95% CI 1.77–20.35), pN2–pN3 (hazard ratio 7.95, 95% CI 2.31–27.37), the presence of BVI (hazard ratio 2.88, 95% CI 1.35–6.13) and triple-negative status (hazard ratio 4.25, 95% CI 2.08–8.72).

For patients aged <35, multivariate analyses indicated that positive axillary lymph nodes (pN1–pN3) and triple-negative status were the independent factors associated with poor DFS and OS (Table 4). For those aged 35–39, triple-negative status was the only independent adverse prognostic factor identified. Axillary lymph node status was not found to be an independent factor, probably due to the

Table 1. Clinicopathological characteristics of breast cancer patients under 40 years old ($n = 242$)

Variable	All patients		Aged <35		Aged 35–39		P^a
	($n = 242$)	(%)	($n = 99$)	(%)	($n = 143$)	(%)	
Familial breast cancer							
No	192	79.3	78	78.8	114	79.7	
Yes	50	20.7	21	21.2	29	20.3	0.492
Primary tumor							
pT1	25	10.3	11	11.1	14	9.8	
pT2	78	32.2	37	37.4	41	28.7	
pT3	112	46.3	40	40.4	72	50.3	
pT4	27	11.1	11	11.1	16	11.2	0.436
Regional lymph node							
pN0	127	52.5	55	55.5	72	50.3	
pN1	65	26.9	21	21.2	44	30.8	
pN2	34	14.0	16	16.2	18	12.6	
pN3	16	6.6	7	7.1	9	6.3	0.411
Histological type							
Invasive ductal carcinoma	221	91.3	95	96.0	126	88.1	
Invasive lobular carcinoma	5	2.1	1	1.0	4	2.8	
Others	16	6.6	3	3.0	13	9.0	0.103
Histological grade							
Grade 1	14	5.8	3	3.0	11	7.7	
Grade 2	84	34.8	31	31.3	53	37.1	
Grade 3	144	59.5	65	65.7	79	55.2	0.148
Lymph vessel invasion							
Absent	98	40.5	46	46.5	52	36.4	
Focal–moderate	135	55.8	50	50.5	85	59.4	
Extensive	9	3.7	3	3.0	6	4.2	0.283
Blood vessel invasion							
Absent	222	91.7	89	89.9	133	93.0	
Present	20	8.3	10	10.1	10	7.0	0.264
Estrogen receptor							
Negative	78	32.2	35	35.4	43	30.1	
Positive	164	67.8	64	64.6	100	69.9	0.234
Progesterone receptor							
Negative	63	26.0	34	34.3	29	20.3	
Positive	179	74.0	65	65.7	114	79.7	0.014
HER2 receptor							
Negative	203	83.9	81	81.8	122	85.3	
Positive	39	16.1	18	18.2	21	14.7	0.290
Subtype							
HR+HER2−	167	69.0	61	61.6	106	74.1	
HR+HER2+	25	10.3	11	11.1	14	9.8	
HR−HER2+	14	5.8	7	7.0	7	4.9	

Continued

Table 1. *Continued*

Variable	All patients		Aged <35		Aged 35–39		P^a
	($n = 242$)	(%)	($n = 99$)	(%)	($n = 143$)	(%)	
HR−HER2− (triple-negative)	36	14.9	20	20.3	16	11.2	0.165
Operative procedure							
Breast-conserving surgery	87	36.0	40	40.4	47	32.9	
Mastectomy	155	64.0	59	59.6	96	67.1	0.230
Radiation							
No	163	67.4	66	66.6	97	67.8	
Yes	79	32.6	33	33.3	46	32.2	0.479
Adjuvant endocrine therapy							
No	84	34.7	38	38.4	46	32.2	
Yes	158	65.3	61	61.6	97	67.8	0.318
Adjuvant chemotherapy							
No	89	36.8	35	35.4	54	37.8	
Yes	153	63.2	64	64.6	89	62.2	0.702
DFS event							
None	156	64.5	68	68.7	88	61.5	
Second primary breast cancer	9	3.7	4	4.0	5	3.5	
Locoregional relapse	18	7.4	8	8.1	10	7.0	
Distant relapse—non-visceral	21	8.7	5	5.1	16	11.2	
Distant relapse—visceral	38	15.7	14	14.1	24	16.8	0.493

[a]χ^2 test.
HR, hormone receptor; DFS, disease-free survival.

subtraction of LVI and BVI, which significantly correlate with positive axillary lymph nodes (Table 4).

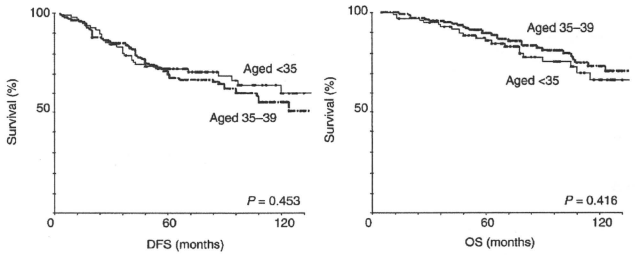
DISCUSSION

Although being 'young' has been reported to be a predictor of poor prognosis independent of other known factors (17–21), the definition of 'young' has varied across studies. The age of 35 years has been used as a cutoff age based on consensus in the international guidelines for treatment of primary breast cancer (1–5). However, the St Gallen international expert consensus panel discontinued the use of the threshold of 35 years of age as a risk category in 2009 (22).

The primary objective of this study was to verify whether breast cancer patients aged <35 at diagnosis have poorer prognoses than those aged 35–39 or to identify the prognostic value of age in younger premenopausal patients under 40 years old. Our results did not indicate any significant differences between patients aged <35 years and those aged 35–39 years in either DFS or OS, and age at diagnosis was not an independent factor associated with DFS or OS in our cohort of breast cancer patients younger than 40 years. We believe that these observations are reliable because the distribution of various clinical and pathological factors did not differ significantly between the two age groups.

A population-based study in Switzerland found no effect of young age on survival when accounting for breast tumor characteristics and treatment (23). A study by van de Vijver et al. (6) also demonstrated that, whereas gene-expression profile was a powerful predictor of disease outcome in younger women with breast cancer, age was not an independent prognostic factor. Younger premenopausal women have been reported to more frequently present with breast cancer marked by poor prognostic features such as higher T stage, positive lymph nodes, endocrine non-responsiveness, high grade, extensive PVI and high proliferating fraction than older premenopausal women (24–29). Kollias et al. (25) concluded that age itself had no influence on the prognosis of individuals because the association of poor prognosis with young age at diagnosis could be explained by a higher proportion of aggressive tumors.

Our present study of breast cancer patients under the age of 40 supports these observations and we consider that the age of <35 years at diagnosis is an unreasonable threshold to identify patients with primary breast cancer at high risk of relapse.



	5-year survival	10-year survival
Aged <35	72.9%	60.2%
Aged 35–39	70.1%	55.3%

	5-year survival	10-year survival
Aged <35	86.1%	66.9%
Aged 35–39	89.7%	73.9%

Figure 1. Kaplan–Meier curves of disease-free survival (DFS) and overall survival (OS) compared between breast cancer patients aged <35 years ($n = 99$) and aged 35–39 years ($n = 143$).

Table 2. Univariate and multivariate analyses of clinicopathological factors associated with disease-free survival in breast cancer patients under 40 years old ($n = 242$)

Variable	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	P^a	Hazard ratio	95% CI	P^a
Age						
<35	1	–	–	1	–	–
35–39	1.18	0.76–1.84	0.455	1.27	0.80–2.02	0.320
Regional lymph node						
pN0	1	–	–	1	–	–
pN1	2.93	1.69–5.10	<0.001	3.69	1.61–8.47	0.002
pN2–3	6.23	3.67–10.57	<0.001	6.55	2.72–15.75	<0.001
Lymph vessel invasion						
Absent	1	–	–	1	–	–
Focal–moderate	3.32	1.86–5.90	<0.001	2.29	1.19–4.38	0.013
Extensive	4.90	2.64–9.11	<0.001	2.10	0.95–4.65	0.066
Blood vessel invasion						
Absent	1	–	–	1	–	–
Present	3.90	2.23–6.84	<0.001	1.99	1.05–3.78	0.034
Subtype						
HR+HER2–	1	–	–	1	–	–
HR+HER2+	1.22	0.62–2.40	0.559	1.12	0.53–2.36	0.768
HR–HER2+	0.89	0.32–2.46	0.822	1.11	0.39–3.15	0.847
HR–HER2– (triple-negative)	2.16	1.25–3.73	0.006	2.45	1.37–4.36	0.002

95% CI, 95% confidence interval; HR, hormone receptor.
[a]Cox proportional hazards model.

Table 3. Univariate and multivariate analyses of clinicopathological factors associated with overall survival in breast cancer patients under 40 years old (n = 242)

Variable	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	P^a	Hazard ratio	95% CI	P^a
Age						
<35	1	–	–	1	–	–
35–39	0.80	0.46–1.34	0.418	0.86	0.47–1.57	0.617
Regional lymph node						
pN0	1	–	–	1	–	–
pN1	4.90	2.13–11.28	<0.001	6.00	1.77–20.35	0.004
pN2–3	10.47	4.72–23.24	<0.001	7.95	2.31–27.37	0.001
Lymph vessel invasion						
Absent	1	–	–	1	–	–
Focal–moderate	4.22	1.82–9.77	0.001	2.41	0.98–5.98	0.057
Extensive	7.71	3.23–18.41	<0.001	2.80	0.95– 8.26	0.063
Blood vessel invasion						
Absent	1	–	–	1	–	–
Present	5.69	3.02–10.73	0.077	2.88	1.35–6.13	0.006
Subtype						
HR+HER2–	1	–	–	1	–	–
HR+HER2+	0.92	0.32–2.62	0.876	0.73	0.24–2.22	0.584
HR–HER2+	1.33	0.41–4.38	0.636	1.64	0.46–5.85	0.445
HR–HER2– (triple-negative)	3.65	1.92–6.95	<0.001	4.25	2.08–8.72	<0.001

95% CI, 95% confidence interval; HR, hormone receptor.
[a]Cox proportional hazards model.

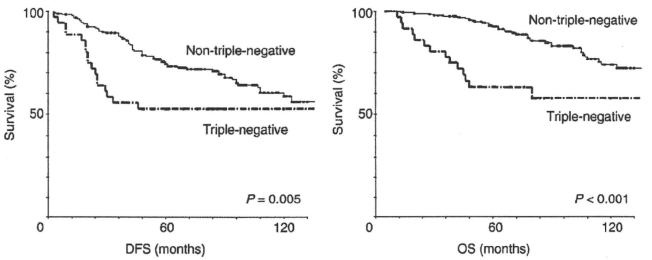
In contrast to our findings, de la Rochefordiere et al. (19) reported that, in a series of 1703 patients from a single institution, the relationship between recurrence hazard and age was best fitted by a log-linear function that indicated a 4% decrease in recurrence and a 2% decrease in death for every year of age in premenopausal women. Han and Kang also recently reported that in patients younger than 35 years, the risk of death rose by 5% for every year of decrease in age, whereas death risk did not vary significantly with age in patients aged 35 years or older (30).

What is more, our unpublished data confirms that breast cancer patients aged <40 years have poorer DFS than those aged 41–49 years (5-year DFS: 79 vs. 86%, $P = 0.04$), while no significant difference was found in OS (5-year OS: 86 vs. 90%, $P = 0.2$). However, there were a much greater number of patients aged 41–49 years compared with those aged <40 years, and the difference in sample number between the two groups was beyond the allowed limit. Therefore, we limited ourselves only to calculating DFS and OS for patients between 40 and 49 years of age. Anders et al. (31) documented similar findings that survival rate in patients who were diagnosed before the age of 40 years was worse when compared with that in older women.

These results indicate that age does have some impact on long-term outcome of patients. Our report and unpublished data suggest that other clinicopathological features rather than age at diagnosis should be used to determine individualized treatment courses for breast cancer patients under 40 years old, but not across all age groups. Further analyses are needed in order to assess the prognostic value of age at diagnosis in women with primary breast cancer across all age groups. However, this can still be a significant finding given that women are now commonly bearing children at older ages in Japan.

Our secondary objective in this study was to assess prognostic factors specific for younger premenopausal women with primary breast cancer. We found that the most important factors associated with poor DFS and OS in patients under the age of 40 were positive axillary lymph nodes (pN1–pN3) and triple-negative status. Triple-negative status was also an independent factor associated with worse DFS and OS in both age groups.

Previous studies have identified axillary lymph node status, HR and HER2 status, tumor size, histological grade, operative procedure, radiation therapy, adjuvant systemic therapy, family history of ovarian cancer and age <35 or 40



	5-year survival	10-year survival
Non-triple-negative	74.5%	58.8%
Triple-negative	52.6%	52.6%

	5-year survival	10-year survival
Non-triple-negative	92.7%	74.3%
Triple-negative	63.0%	57.8%

Figure 2. Kaplan–Meier curves of DFS and OS compared between triple-negative breast cancer patients ($n = 36$) and breast cancer patients whose tumors fall into one of the other three subtypes (non-triple-negative; $n = 206$).

Table 4. Multivariate analyses of clinicopathological factors associated with disease-free survival and overall survival for the two age groups; aged <35 vs. aged 35–39

Variable	Disease-free survival						Overall survival					
	Aged <35 ($n = 99$)			Aged 35–39 ($n = 143$)			Aged <35 ($n = 99$)			Aged 35–39 ($n = 143$)		
	Hazard ratio	95% CI	P^a	Hazard ratio	95% CI	P^a	Hazard ratio	95% CI	P^a	Hazard ratio	95% CI	P^a
Regional lymph node												
pN0	1	–	–	1	–	–	1	–	–	1	–	–
pN1	18.64	3.36–103.30	0.001	1.43	0.52–3.94	0.489	56.57	7.74–413.30	<0.001	1.30	0.32–5.37	0.715
pN2–3	11.86	1.95–72.00	0.007	3.72	1.28–10.83	0.016	52.95	5.55–505.71	0.001	1.94	0.46–8.25	0.368
Lymph vessel invasion												
Absent	1	–	–	1	–	–	1	–	–	1	–	–
Focal–moderate	1.82	0.62–5.32	0.277	2.32	1.00–5.40	0.051	0.86	0.24–3.12	0.816	6.06	1.22–30.10	0.028
Extensive	3.36	0.76–14.83	0.110	2.04	0.75–5.51	0.162	2.69	0.50–14.55	0.250	4.49	0.83–24.42	0.082
Blood vessel invasion												
Absent	1	–	–	1	–	–	1	–	–	1	–	–
Present	2.57	0.90–7.29	0.077	1.32	0.53–3.32	0.555	2.75	0.81–9.32	0.104	3.28	1.07–10.06	0.037
Subtype												
HR+HER2−	1	–	–	1	–	–	1	–	–	1	–	–
HR+HER2+	1.04	0.31–3.43	0.951	1.00	0.33–3.02	0.996	0.49	0.09–2.67	0.407	0.66	0.12–3.69	0.640
HR−HER2+	1.86	0.38–9.17	0.447	0.74	0.16–3.42	0.703	1.17	0.11–12.66	0.899	1.35	0.22–8.25	0.745
HR−HER2−(triple-negative)	3.80	1.39–10.42	0.009	3.16	1.42–7.01	0.005	7.58	2.18–26.37	0.001	7.64	2.66–21.94	<0.001

95% CI, 95% confidence interval; HR, hormone receptor.
[a]Cox proportional hazards model.

years as independent prognostic factors in younger premenopausal patients (17,19–21,23,24,27,28).

Axillary lymph node status in particular has been highlighted as a powerful independent prognostic parameter in women with primary breast cancer across all age groups. However, in the present study, axillary lymph node status was not an independent prognostic factor in patients aged 35–39 years. This discrepancy with previous studies is likely the result of the subtraction effects of LVI and BVI, which significantly correlate with positive axillary lymph nodes. We also observed that, in univariate analyses, patients who were treated with chemotherapy had significantly worse DFS and OS. This finding reflects the significantly higher proportion of positive axillary lymph nodes in those patients. Taken together, these results support axillary lymph node status as an important prognostic factor.

The triple-negative subtype or the basal-like subtype (defined immunohistochemically as ER negative, HER2 negative and cytokeratin 5/6 and/or HER1 positive) (32) is associated with aggressive histology and poor clinical outcome. In our study, triple-negative status was confirmed as a prognostic factor for poorer long-term outcome. The triple-negative subtype accounts for ~15% of the four tumor subtypes in the general population and for a higher percentage of breast cancer arising in African-American women (33,34) which is a contributing factor to their poorer prognosis (9). According to surveillance data from the Registration Committee of the Japanese Breast Cancer Society, the triple negative subtype accounts for 15.5% of breast cancers, with no difference in mean age at diagnosis among the four tumor subtypes (35). In our study of breast cancer patients under age 40, the proportion of patients falling into each of these four tumor subtypes was approximately the same as that in a representative population of Japanese women with breast cancer, and did not differ significantly between patients aged <35 and those aged 35–39 years. Further studies are needed to clarify the associations between the factors involved in triple-negative status, younger onset and poorer prognosis in patients with breast cancer.

In conclusion, our results did not indicate any significant differences between patients aged <35 years and those aged 35–39 years in either DFS or OS. In our cohort of breast cancer patients under the age of 40, the independent factors associated with poor DFS and OS included positive axillary lymph nodes (pN1–pN3) and triple-negative status, but not age at diagnosis. Adverse prognostic factors also did not differ considerably between the two age groups. Our findings suggest that other clinicopathological features rather than age should be used to determine individualized treatment courses for breast cancer patients younger than 40 years.

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Conflict of interest statement

None declared.

References

1. Goldhirsch A, Glick JH, Gelber RD, Senn HJ. Meeting highlights: international consensus panel on the treatment of primary breast cancer. *J Natl Cancer Inst* 1998;90:1601–8.
2. Goldhirsch A, Glick JH, Gelber RD, Coates AS, Senn HJ. Meeting highlights: international consensus panel on the treatment of primary breast cancer. Seventh international conference on adjuvant therapy of primary breast cancer. *J Clin Oncol* 2001;19:3817–27.
3. Goldhirsch A, Wood WC, Gelber RD, Coates AS, Thurlimann B, Senn HJ. Meeting highlights: updated international expert consensus on the primary therapy of early breast cancer. *J Clin Oncol* 2003;21:3357–65.
4. Goldhirsch A, Glick JH, Gelber RD, Coates AS, Thurlimann B, Senn HJ. Meeting highlights: international expert consensus on the primary therapy of early breast cancer 2005. *Ann Oncol* 2005;16:1569–83.
5. Goldhirsch A, Wood WC, Gelber RD, Coates AS, Thurlimann B, Senn HJ. Progress and promise: highlights of the international expert consensus on the primary therapy of early breast cancer 2007. *Ann Oncol* 2007;18:1133–44.
6. van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, et al. A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* 2002;347:1999–2009.
7. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA* 2001;98:10869–74.
8. Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci USA* 2003;100:8418–23.
9. Carey LA, Perou CM, Livasy CA, Dressler LG, Cowan D, Conway K, et al. Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. *J Am Med Assoc* 2006;295:2492–502.
10. Bland KI, Copeland EM, III. The breast: comprehensive management of benign and malignant diseases. In: Lynch HT, Marcus JN, Lynch J, Snyder CL, Rubinstein WS, editors. *Breast Cancer Genetics: Syndromes, Genes, Pathology, Counseling, Testing, and Treatment*. 4th edn. Philadelphia: Saunders Elsevier 2009;375.
11. Kinoshita T, Fukutomi T, Iwamoto E, Akashi-Tanaka S. Prognosis of breast cancer patients with familial history classified according to their menopausal status. *Breast J* 2004;10:218–22.
12. Sobin LH, Wittekind C. *TNM Classification of Malignant Tumours*. 6th edn. New York: Wiley-Liss 2002;131–41.
13. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* 1991;19:403–10.
14. Kurosumi M. Immunohistochemical assessment of hormone receptor status using a new scoring system (J-Score) in breast cancer. *Breast Cancer* 2007;14:189–93.
15. Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *J Clin Oncol* 2007;25:118–45.
16. Colleoni M, Rotmensz N, Maisonneuve P, Sonzogni A, Pruneri G, Casadio C, et al. Prognostic role of the extent of peritumoral vascular invasion in operable breast cancer. *Ann Oncol* 2007;18:1632–40.
17. Albain KS, Allred DC, Clark GM. Breast cancer outcome and predictors of outcome: are there age differentials? *J Natl Cancer Inst Monogr* 1994;35–42.
18. Walker RA, Lees E, Webb MB, Dearing SJ. Breast carcinomas occurring in young women (<35 years) are different <35 years) are different. *Br J Cancer* 1996;74:1796–800.
19. de la Rochefordiere A, Asselain B, Campana F, Scholl SM, Fenton J, Vilcoq JR, et al. Age as prognostic factor in premenopausal breast carcinoma. *Lancet* 1993;341:1039–43.

20. Han W, Kim SW, Park IA, Kang D, Youn YK, Oh SK, et al. Young age: an independent risk factor for disease-free survival in women with operable breast cancer. *BMC Cancer* 2004;4:82.
21. Elkum N, Dermime S, Ajarim D, Al-Zahrani A, Alsayed A, Tulbah A, et al. Being 40 or younger is an independent risk factor for relapse in operable breast cancer patients: the Saudi Arabia experience. *BMC Cancer* 2007;7:222.
22. Goldhirsch A, Ingle JN, Gelber RD, Coates AS, Thurlimann B, Senn HJ. Thresholds for therapies: highlights of the St Gallen International Expert Consensus on the primary therapy of early breast cancer 2009. *Ann Oncol* 2009;20:1319–29.
23. Rapiti E, Fioretta G, Verkooijen HM, Vlastos G, Schafer P, Sappino AP, et al. Survival of young and older breast cancer patients in Geneva from 1990 to 2001. *Eur J Cancer* 2005;41:1446–52.
24. Nixon AJ, Neuberg D, Hayes DF, Gelman R, Connolly JL, Schnitt S, et al. Relationship of patient age to pathologic features of the tumor and prognosis for patients with stage I or II breast cancer. *J Clin Oncol* 1994;12:888–94.
25. Kollias J, Elston CW, Ellis IO, Robertson JF, Blamey RW. Early-onset breast cancer—histopathological and prognostic considerations. *Br J Cancer* 1997;75:1318–23.
26. Colleoni M, Rotmensz N, Robertson C, Orlando L, Viale G, Renne G, et al. Very young women (<35 years) with operable breast cancer: features of disease at presentation <35 years) with operable breast cancer: features of disease at presentation. *Ann Oncol* 2002;13:273–9.
27. Zabicki K, Colbert JA, Dominguez FJ, Gadd MA, Hughes KS, Jones JL, et al. Breast cancer diagnosis in women < or = 40 versus 50 to 60 years: increasing size and stage disparity compared with older women over time < or =40 versus 50 to 60 years: increasing size and stage disparity compared with older women over time. *Ann Surg Oncol* 2006;13:1072–7.
28. Kim JK, Kwak BS, Lee JS, Hong SJ, Kim HJ, Son BH, et al. Do very young Korean breast cancer patients have worse outcomes? *Ann Surg Oncol* 2007;14:3385–91.
29. Gonzalez-Angulo AM, Broglio K, Kau SW, Eralp Y, Erlichman J, Valero V, et al. Women age < or = 35 years with primary breast carcinoma: disease features at presentation < or = 35 years with primary breast carcinoma: disease features at presentation. *Cancer* 2005;103:2466–72.
30. Han W, Kang SY. Relationship between age at diagnosis and outcome of premenopausal breast cancer: age less than 35 years is a reasonable cut-off for defining young age-onset breast cancer. *Breast Cancer Res Treat* 119:193–200.
31. Anders CK, Johnson R, Litton J, Phillips M, Bleyer A. Breast cancer before age 40 years. *Semin Oncol* 2009;36:237–49.
32. Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, et al. Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. *Clin Cancer Res* 2004;10: 5367–74.
33. Ihemelandu CU, Leffall LD, Jr, Dewitty RL, Naab TJ, Mezghebe HM, Makambi KH, et al. Molecular breast cancer subtypes in premenopausal African-American women, tumor biologic factors and clinical outcome. *Ann Surg Oncol* 2007;14:2994–3003.
34. Stead LA, Lash TL, Sobieraj JE, Chi DD, Westrup JL, Charlot M, et al. Triple-negative breast cancers are increased in black women regardless of age or body mass index. *Breast Cancer Res* 2009;11:R18.
35. Iwase H, Kurebayashi J, Tsuda H, Ohta T, Kurosumi M, Miyamoto K, et al. Clinicopathological analyses of triple negative breast cancer using surveillance data from the Registration Committee of the Japanese Breast Cancer Society. *Breast Cancer* 2010;17:118–24.

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CLINICAL TRIAL

Tumor-infiltrating lymphocytes are correlated with response to neoadjuvant chemotherapy in triple-negative breast cancer

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Abstract The purpose of the present study was to identify histological surrogate predictive markers of pathological complete response (pCR) to neoadjuvant chemotherapy (NAC) in triple-negative breast cancer (TNBC). Among 474 patients who received NAC and subsequent surgical therapy for stage II–III invasive breast carcinoma between 1999 and 2007, 102 (22%) had TNBC, and 92 core needle biopsy (CNB) specimens obtained before NAC were available. As controls, CNB specimens from 42 tumors of the hormone receptor-negative and HER2-positive (HR−/HER2+) subtype and 46 tumors of the hormone receptor-positive and HER2-negative (HR+/HER2−) subtype were also included. Histopathological examination including tumor-infiltrating lymphocytes (TIL) and tumor cell apoptosis, and immunohistochemical studies for basal markers were performed, and the correlation of these data with pathological therapeutic effect was analyzed. The rates of pCR at the primary site were higher for TNBC (32%) and the HR−/HER2+ subtype (21%) than for the HR+/HER2− subtype (7%) ($P = 0.006$). Expression of basal markers and p53, histological grade 3, high TIL scores, and apoptosis were more frequent in TNBC and the HR−/HER2+ subtype than in the HR+/HER2− subtype ($P = 0.002$ for TIL and $P < 0.001$ for others). In TNBC, the pCR rates of tumors showing a high TIL score and of those showing a high apoptosis score were 37 and 47%, respectively, and significantly higher or tended to be higher than those of the tumors showing a low TIL score and of the tumors showing a low apoptosis score (16 and 27%, respectively, $P = 0.05$ and 0.10). In a total of 180 breast cancers, the pCR rates of the tumors showing a high TIL score (34%) and of those showing a high apoptosis score (35%) were significantly higher than those of the tumors showing a low TIL score (10%) and those of the tumors showing a low apoptosis score (19%) ($P = 0.0001$ and 0.04, respectively). Histological grade and basal marker expression were not correlated with pCR. Although the whole analysis was exploratory, the degree of TIL correlated with immune response appear to play a substantial role in the response to NAC in TNBC.

Keywords Triple-negative breast cancer · Neoadjuvant chemotherapy · Pathological complete response · Tumor-infiltrating lymphocytes · Tumor cell apoptosis

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Introduction

The heterogeneous nature of breast cancer has been demonstrated by gene expression profiling using the DNA microarray technique [1–3]. Genetically, invasive breast cancers have been classified into distinct intrinsic subtypes comprising luminal A, luminal B, ERBB2 (HER2), basal-like, and normal breast subtypes [1–3], which demonstrate characteristic immunohistochemical features and clinical behavior [4–8]. Both basal-like and normal breast subtypes are immunohistochemically characterized by lack of expression of the estrogen receptor (ER), progesterone receptor (PgR), and HER2, and thus are also categorized as triple-negative breast cancer (TNBC). TNBC, which accounts for 10–15% of all breast cancers, tends to show visceral metastasis and aggressive clinical behavior [9].

TNBC is unresponsive to specific targeted therapies such as trastuzumab for HER2-positive breast cancer, or hormonal therapy for hormone-receptor-positive breast cancer. In cases of operable TNBC, only systemic chemotherapy has been shown to be effective in an adjuvant or neoadjuvant setting. Although patients with TNBC are more likely to achieve a pathological complete response (pCR) after neoadjuvant chemotherapy (NAC) than patients with the luminal subtypes, and pCR is correlated with an excellent clinical outcome, TNBC patients with residual disease after NAC have a poor prognosis [10, 11]. However, the factor that determines sensitivity to chemotherapy in patients with TNBC is uncertain.

TNBC itself may show heterogeneous characteristics including basal-like and normal breast subtypes, as judged from gene expression profiles [1–3]. Accordingly, it is important to investigate the pathological factors associated with response to chemotherapy in patients with TNBC.

The aim of the present study was to identify the factors that predict pCR after NAC in patients with TNBC by examination of histological parameters including histological grade and type, the presence of tumor-infiltrating lymphocytes (TIL), and tumor cell apoptosis, as well as immunohistochemical parameters including basal-like markers and p53.

Materials and methods

Patients and tissue samples

Among 474 patients who received NAC and subsequent surgical therapy for stage II–III invasive breast carcinoma between 1999 and 2007, 102 (22%) had TNBC. Originally, we planned to compare 100 TNBCs with 100 non-TNBCs as controls on the basis of matching for age (±5years) and clinical stage (II and III). In the 100 control cases, we planned to include 50 cases of the HR−/HER2+ subtype (HER2 positive and ER/PgR negative in routine immunohistochemistry) and 50 cases of the HR+/HER2− subtype (ER and/or PgR positive but HER2 negative in routine immunohistochemistry). From these patients, sufficient CNB specimens before NAC were available from 92 tumors of TNBC, 42 tumors of the HR−/HER2+ subtype, and 46 tumors of the HR+/HER2− subtype. Clinical characteristics of all patients were obtained from the medical records. All patients received neoadjuvant anthracycline-based regimens (adriamycin 60 mg/m^2 plus cyclophosphamide 600 mg/m^2 (AC) or cyclophosphamide 600 mg/m^2 plus epirubicin 100 mg/m^2/5-fluorouracil 600 mg/m^2 (CEF)) alone, taxane-based regimens (weekly paclitaxel 80 mg/m^2, or triweekly docetaxel 75 mg/m^2) alone, or anthracycline and taxane sequentially or concurrently (adriamycin 50 mg/m^2 plus docetaxel 60 mg/m^2 (AT), AC or CEF followed by weekly paclitaxel or triweekly docetaxel). Trastuzumab was not used for the 42 patients with tumors of HR−/HER2+ subtype, because the use of trastuzumab for neoadjuvant therapy of primary breast cancer was not approved in Japan. The patients have been followed up for 64.8 months on an average (7.2–138.2 months). All specimens were formalin-fixed and paraffin-embedded, and 4-μm-thick sections were prepared for hematoxylin and eosin staining and immunohistochemistry (IHC) and were reviewed by two observers including an experienced pathologist (T.H.). The present study was approved by the Institutional Review Board of the National Cancer Center.

Histopathological evaluation

Pathological therapeutic effect was assessed for resected primary tumors after NAC. Pathological complete response (pCR) was defined as the absence of all invasive disease in the breast tumor according to the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-18 protocol [12]. In addition, we defined quasi-pCR (QpCR) as the absence of invasive tumor or only focal residual invasive carcinoma cells in the primary site [13]. In Japan, Breast Cancer Research Group (JBCRG) 01 study, QpCR after NAC was shown to be correlated with better patient prognosis in comparison with non-QpCR [13]. Furthermore, we took into consideration both the pCR in the primary tumor and no residual tumor in axillary lymph nodes as another classification for histopathological therapeutic effect [14, 15].

Histopathological assessment of predictive factors was made for CNB specimens. Histopathological parameters examined included histological grade [16], histological type [17], presence of tumor-infiltrating lymphocytes (TIL), apoptosis, and correlation of these parameters with intrinsic subtypes and pCR. Histological grade was assigned on the basis of the criteria of Elston and Ellis.

For the evaluation of TIL, both areas of stroma infiltrated by lymphocytes (proportional score) and intensity of lymphatic infiltration (intensity score) were taken into consideration. Proportional scores were defined as 3, 2, 1, and 0 if the area of stroma with lymphoplasmacytic infiltration around invasive tumor cell nests were >50, >10–50, ≤10%, and absent, respectively. Intensity scores were defined as 2, 1, and 0, if the intensity of lymphatic infiltration was marked, mild, and absent, respectively (Fig. 1). Lymphocyte infiltration surrounding non-invasive tumor cells was not taken into account. The proportional and intensity scores were summed for each tumor, and the TIL score was classified as high if the sum was 3–5, whereas the TIL score was classified as low if the sum was 0–2. As criteria for apoptosis, scores were defined as 2, 1, and 0 if apoptotic cells (arrows in Fig. 2) were>10 per 10 high-power fields (HPFs) using 40× objective lens, 5–9 per 10 HPFs, and less than 5 per 10 HPFs, respectively.

Immunohistochemistry (IHC)

IHC was performed for CNB specimens using the following primary antibodies: anti-ER (clone 1D5; Dako), anti-PgR (clone PgR636; Dako), anti-HER2 (polyclonal, HercepTest II, Dako), anti-p53 (clone DO-7; Dako), anti-cytokeratin (CK) 5/6 (clone D5/16 B4; Dako), anti-CK14 (NCL-LL002, Novocastra), and anti-EGFR (pharmDX, clone 2-18C9, Dako).

Because ER, PgR, and HER2 tests had been performed by various antibodies and methods, these tests were re-tested again according to standardized antibodies and methods in the present study. The sections were deparaffinized, subjected to antigen retrieval by incubating in target retrieval solution, high pH (Dako) for 40 min at 95°C for ER and PgR, in sodium citrate buffer (pH 6.0) with a microwave oven for 15 min at 97°C for CK14, in sodium citrate buffer (pH 6.0) with a water bath for 15 min at 98°C for CK5/6, or by autoclaving in sodium citrate buffer (pH 6.0) for 20 min at 121°C for p53, then allowed to cool at room temperature. Endogenous peroxidase and non-specific staining were blocked in 2% normal swine serum (Dako). The slides were incubated with primary antibodies at 4°C overnight and then reacted with a dextran polymer reagent combined with secondary antibodies and peroxidase (Envision Plus, Dako) for 2 h at room temperature. Specific antigen–antibody reactions were visualized using 0.2% diaminobenzidine tetrahydrochloride and hydrogen peroxide. Counterstaining was performed using Mayer's hematoxylin. For the HER2 and EGFR kits, immunohistochemistry was performed in accordance with the protocol recommended by the manufacturer.

ER and PgR were judged as positive if the Allred score was ≥3 and as negative if the Allred score was ≤2 [18]. HER2 protein overexpression was judged as positive when the score was 3+, equivocal when the score was 2+, and negative when the score was 0 or 1+ in accordance with the ASCO/CAP recommendation [19]. TNBC was defined as negative for ER, PgR, and HER2, while the HR+/HER2− subtype was defined as positive for ER or PgR and negative for HER2, and the HR−/HER2+ subtype was defined as negative for ER and PgR, and positive for HER2. The basal-like subtype was defined as CK5/

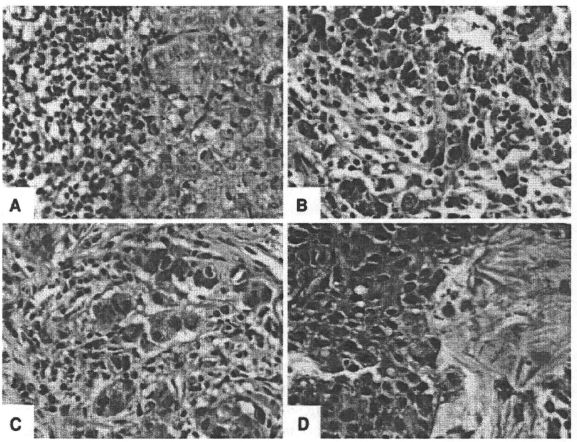

Fig. 1 Histophathological features of tumor-infiltrating lymphocytes (TILs). **a** High TIL score (proportional score 3+ intensity score 2); **b** High TIL score (proportional score 2+ intensity score 2); **c** Low TIL score (proportional score 1+ intensity score 2); **d** Low TIL score (proportional score 0, intensity score 0). Original magnification: 400×