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EPIDEMIOLOGY

Impact of recent parity on histopathological tumor features and breast cancer outcome in premenopausal Japanese women

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Abstract Although previous studies have reported that onset at young age is associated with poor prognosis in breast cancer, the correlation between reproductive factors, breast cancer characteristics, and prognosis remains unclear. Five hundred and twenty-six premenopausal young women diagnosed with primary invasive breast cancer between January 2000 and December 2007 were included in this study. Patients were classified into four groups according to their reproductive history: women who gave birth within the previous 2 years (group A), women who gave birth between 3 and 5 years previously (group B), women who gave birth more than 5 years previously (group C), and nulliparous women (group N). The correlation between the time since last childbirth to diagnosis, histopathological tumor features, and breast cancer prognosis was evaluated. Breast cancer patients who had given birth more recently had more advanced stage tumors; larger sized tumors; a higher rate of axillary lymph node metastases; a higher histological tumor grade; and increased progesterone receptor (PgR)−, HER2+, and triple negative tumors than patients who had given birth less recently or not at all. Group A patients had significantly shorter survival times than patients in both groups C and N (log rank test; $p < 0.001$). After adjusting for tumor characteristics, the hazard ratio for death in group A was 2.19 compared with group N ($p = 0.036$), and the adjusted hazard ratio restricted to patients in group A with hormone-receptor-positive, and HER2− tumors was 3.07 ($p = 0.011$). Young breast cancer patients who had given birth more recently had tumors with more aggressive features and worse prognoses compared with patients who had given birth less recently or were nulliparous.

Keywords Reproductive history · Subtype · Prognosis · Breast cancer in young women

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Introduction

Many studies have reported that young breast cancer patients have a poor prognosis [1–4]; however, the value of age as a prognostic factor remains a matter of debate [5]. Epidemiological studies have suggested that endogenous host environments, such as reproductive history, body-mass index, and BRCA germline mutation, may correlate with breast cancer features and prognosis [6–14]. In addition, molecular subtypes are known to be associated with survival [15–17], although the correlation between host environments, including reproductive factors and molecular subtype, remains unclear. Our objective was to explore the impact of host-related factors on the histopathological tumor features and prognosis in breast cancer patients.

Patients and methods

Patients

All premenopausal women of 20–44 years of age diagnosed with primary invasive breast cancer between January 2000 and December 2007 at the National Cancer Center Hospital in Tokyo (526 patients) were included in the present study. Clinical and pathological information was retrieved from medical charts. The follow-up period was completed in December 2011, and the median duration of follow-up was 6.3 years (range: 0.1–11.7 years), during which time 90 patients died. This study protocol was approved by the institutional review board at the National Cancer Center Hospital in Tokyo.

Data collection

Data was collected from various sources, including clinical pathology reports and the patients themselves. A questionnaire was routinely used to assess baseline characteristics at the initial visit for all patients. It included host-related factors, such as body-mass index, smoking history, drinking habits, and family history of breast and/or ovarian cancer in first or second-degree relatives (FH), and menstrual and reproductive factors, such as age at menarche, number of pregnancies, number of children, age at first and last delivery, and duration of breastfeeding. Patients were classified into four groups according to their reproductive history: women who gave birth within the previous 2 years (group A), women who gave birth between 3 and 5 years previously (group B), women who gave birth more than 5 years previously (group C), and nulliparous women (group N). Tumor characteristics, including histopathology; estrogen receptor (ER), progesterone receptor (PgR), and human EGFR-related 2 (HER2) statuses; and histological grade were abstracted from the relevant diagnostic pathology reports. Clinical stage was determined according to the TNM clinical classification from the American Joint Committee on Cancer/The International Union Against Cancer (AJCC/UICC) 6th edition.

Breast cancer subtypes were categorized according to expression of ER, PgR, and HER2 determined by immunohistochemistry. Hormone-receptor positivity was defined as positive staining in more than 1 % of the tumor cell nuclei. HER2 positivity was defined as an immunohistochemistry score of 3+ (intense staining of the cell membrane in more than 30 % of the cancer cells) or an IHC score of 2+ and positive fluorescence in situ hybridization (FISH) HER2 amplification signals. Subtypes were defined as follows: HR+HER2−, ER− or PgR+, and HER2−; HR+HER2+, ER− or PgR+, and HER2+; HR−HER2−, ER, PgR−, and HER2− (triple negative); and HR−HER2+, ER− and PgR−, and HER2+ (HER2-enriched).

Statistical analyses

All statistical analyses were performed using SAS Ver. 9.2 statistical software (SAS Statistic Inc., Cary, NC). All the tests were two-sided, and p values of <0.05 were considered significant. For comparison of patient groups, the Chi squared test was used for discrete data, and the Wilcoxon rank sum test was used for continuous data. Overall survival (OS) was calculated from the first day of breast cancer diagnosis until death from any cause. Survival curves were derived from the Kaplan–Meier product limit estimate method, with the log-rank statistic being used to test for differences between groups. Hazard ratios and 95 % confidence intervals (CI) for death were estimated using Cox proportional hazards survival models, with and without adjusting for one or more of the following factors: age at diagnosis, AJCC stage, hormone receptor and HER2 statuses, and histological tumor grade. To determine any trends between age at diagnosis and time from last childbirth to diagnosis, linear regression was used for continuous data, whereas correlation and ANOVA statistics were used for discrete data.

Results

Patient and tumor characteristics

Clinical characteristics at diagnosis according to each group are presented in Table 1. The median age at diagnosis for all patients was 39 years (range: 22–44 years). No difference in the FH of breast cancer was observed between nulliparous and parous women. Among the 526 women included in this study, 37 women (7 %) were classified into

Table 1 Patient characteristics

	Parous			Nulliparous
Time since last parity: Number or patients:	Group A ≤2 years *N* = 37	Group B 3–5 years *N* = 59	Group C >5 years *N* = 181	Group N Nulliparous *N* = 249
Age at diagnosis, median (range)	35 (26–44)	37 (27–43)	41 (32–44)	38 (22–44)
Age at diagnosis category, *N* (%)				
<35	18 (49)	15 (25)	4 (2)	75 (30)
35–39	15 (41)	26 (44)	44 (24)	69 (28)
40–44	4 (11)	18 (31)	133 (73)	105 (42)
Family history of breast and/or ovarian cancer (within second degree), *N* (%)				
Absent	27 (73)	46 (78)	141 (78)	194 (78)
Present	10 (27)	13 (22)	40 (22)	55 (22)
Age at menarche, median (range)	12 (10–15)	12 (10–15)	12 (9–16)	12 (9–16)
Age at first full-term birth, median (range)	30 (23–43)	30 (20–38)	27 (19–38)	
Age at first full-term birth, category, N (%)				
Nulliparous				249 (100)
<30	17 (46)	26 (44)	137 (76)	
≥30	20 (54)	33 (56)	44 (24)	
Number of children, *N* (%)				
0 (nulliparous)				249 (100)
1	19 (51)	21 (36)	52 (29)	
2	11 (30)	29 (49)	105 (58)	
≥3	7 (19)	9 (15)	24 (13)	
Breastfeeding, *N* (%)				
Nulliparous				249 (100)
<6 months	15 (41)	22 (37)	60 (33)	
≥6 months	19 (51)	39 (66)	86 (48)	
Missing data	3 (8)	7 (12)	35 (19)	

group A, 59 (11 %) into group B, 118 (35 %) into group C, and 249 (47 %) into group N. Parous women with breast cancer were much older than nulliparous women, and the trend test showed that age at diagnosis increased as the period from last childbirth increased.

Tumor characteristics at diagnosis according to reproductive history are presented in Table 2. Between nulliparous and parous women, no significant differences were observed in any available factors. However, breast cancer patients who had given birth recently had more advanced stage tumors; larger sized tumors; a higher rate of axillary lymph node metastases; higher histological tumor grade; and more PgR−, HER2+, and triple negative tumors than those who had given birth less recently or not at all.

Impact of the time since last childbirth on outcome

The Kaplan–Meier 5-year OS probability was 64.3 % for group A, 79.3 % for group B, 88.2 % for group C, and 90.6 % for group N. The patients in group A had significantly shorter survival times than patients in both groups C and N (log rank test; $p < 0.001$ for both groups) (Fig. 1). Other host-related factors were not associated with survival.

Using multivariate Cox proportional hazards survival models, survival outcome of young breast cancer patients was associated with AJCC stage, histological tumor grade, and ER status, whereas age at diagnosis and PgR and HER2 statuses were not significantly associated with mortality. Using those models, breast cancer diagnosed within 2 years of last childbirth was an independently poor prognostic factor relative to nulliparity (Table 3). After adjusting for tumor characteristics, the hazard ratio for death in group A was 2.19 (95 % CI, 1.05–4.56; $p = 0.036$), 1.49 in group B (95 % CI, 0.79–2.83; $p = 0.223$), and 0.81 in group C (95 % CI, 0.46–1.43; $p = 0.471$) compared with group N (Table 4; Fig. 2). Among the patients with HR+HER2− tumors, the adjusted hazard ratio for death was 3.07 in group A (95 % CI, 1.30–7.27; $p = 0.011$), 1.01 in group B (95 % CI, 0.39–2.63; $p = 0.977$), and 0.60 in group C

Table 2 Tumor characteristics

Time since last parity:	Parous			Nulliparous	*p* value	
	Group A ≤2 years *N* = 37 *N* (%)	Group B 3–5 years *N* = 59 *N* (%)	Group C >5 years *N* = 181 *N* (%)	Group N nulliparous *N* = 249 *N* (%)	Parous vs. nulliparous	Trend test (parous)
AJCC stage at diagnosis					0.409	0.584
0	1 (3)	1 (2)	4 (2)	8 (3)		
I	5 (13)	16 (27)	52 (29)	60 (24)		
II	18 (49)	26 (44)	97 (53)	140 (56)		
III	9 (24)	14 (24)	21 (12)	34 (14)		
IV	4 (11)	2 (3)	7 (4)	7 (3)		
AJCC T factor at diagnosis					0.679	0.010
Tis	1 (3)	1 (2)	4 (2)	7 (3)		
T1	7 (19)	18 (30)	57 (31)	63 (25)		
T2	16 (43)	23 (39)	90 (50)	130 (52)		
T3	8 (22)	11 (19)	21 (12)	32 (13)		
T4	5 (13)	6 (10)	9 (5)	16 (6)		
T0 (Occult primary)	0 (0)	0 (0)	0 (0)	1 (0)		
Regional lymph node metastasis at diagnosis					0.153	0.005
Negative	19 (51)	37 (63)	133 (73)	184 (74)		
Positive	18 (49)	22 (37)	48 (27)	65 (26)		
Histological type					0.075	0.139
Invasive ductal carcinoma	35 (95)	49 (83)	164 (90)	226 (91)		
Invasive lobular carcinoma	0 (0)	2 (3)	10 (6)	3 (1)		
Others	2 (5)	8 (14)	7 (4)	20 (8)		
Estrogen receptor status					0.436	0.140
Negative	19 (51)	19 (32)	63 (35)	83 (33)		
Positive	18 (49)	39 (66)	117 (64)	165 (67)		
Missing data	0 (0)	1 (2)	1 (1)	1 (0)		
Progesterone receptor status					0.328	0.001
Negative	20 (54)	18 (30)	45 (25)	65 (26)		
Positive	17 (46)	40 (68)	135 (74)	182 (73)		
Missing data	0 (0)	1 (2)	1 (1)	2 (1)		
HER2 status					0.217	0.041
Negative	27 (73)	44 (74)	153 (84)	212 (85)		
Positive	10 (27)	14 (24)	27 (15)	36 (14)		
Missing	0 (0)	1 (2)	1 (1)	1 (0)		
Tumor subtype					0.605	0.004
HR+HER2−	16 (43)	38 (64)	128 (71)	174 (70)		
HR+HER2+	3 (8)	5 (9)	16 (9)	19 (8)		
HR−HER2− (TNBC)	11 (30)	6 (10)	25 (14)	38 (15)		
HR−HER2+	7 (19)	9 (15)	11 (6)	17 (7)		
Missing	0 (0)	1 (2)	1 (1)	1 (0)		
Histological tumor grade					0.253	0.005
Grade 1 and 2	9 (24)	27 (46)	95 (52)	131 (53)		
Grade 3	27 (73)	30 (51)	86 (48)	117 (47)		
Missing data	1 (3)	2 (3)	0 (0)	1 (0)		

AJCC American Joint Committee on Cancer, *HER2* human EGFR-related 2, *HR* hormone receptor, *TNBC* triple negative breast cancer

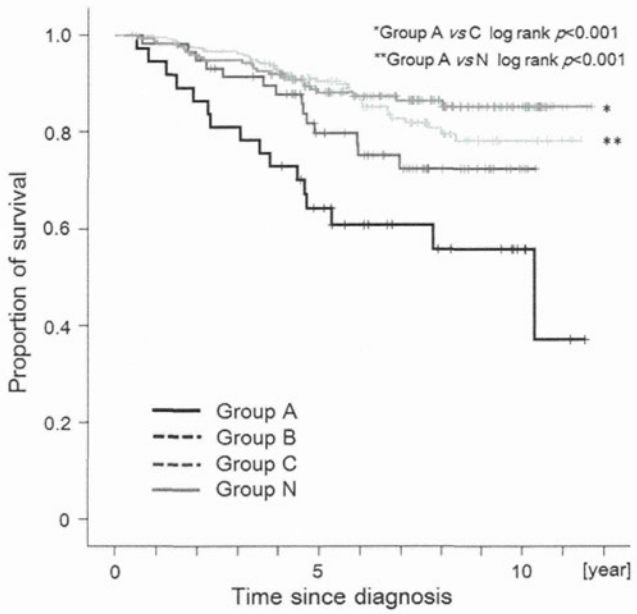

Fig. 1 Kaplan–Meier curves for overall survival based on the time since last childbirth

(95 % CI, 0.26–1.38; $p = 0.228$) compared to group N (Fig. 3a). However, among the patients with other tumor subtypes, no significant differences in survival were observed in any group (Fig. 3b–d). Other multivariate Cox proportional hazard survival models using age at first and last birth, time from first childbirth to diagnosis, or number of children among parous women were not associated with mortality (data not shown).

Discussion

Here, we showed that breast cancer patients with recent parity had shorter survival times than nulliparous patients. Women who had delivered within 2 years of breast cancer diagnosis had tumor(s) at a higher AJCC stage at diagnosis, a lower rate of ER− and PgR+ tumors, a higher rate of HER2+ and triple negative tumors, and a higher histological tumor grade than those with less recent childbirth. Even after adjusting for these well-known prognostic factors, including AJCC stage, hormone receptor and HER2 statuses, and histological tumor grade, women who delivered within 2 years of breast cancer diagnosis had a two-fold increased risk of death (i.e., were twice as likely to die) compared with nulliparous women. Moreover, when the analysis was restricted to patients with HR+HER2− tumors, women with recent parity had an even higher risk of death. Several studies have shown that breast cancer patients with recent childbirth before diagnosis had worse survival outcomes than nulliparous patients or those with a less recent childbirth [18–22]. However, to date, few studies have analyzed the hazard ratio adjusting for not only reproductive factors, but also tumor characteristics [23–26]. This study analyzed the hazard ratio adjusting for both reproductive factors and tumor characteristics, including hormone receptor and HER2 statuses and histological tumor grade.

The patients who were diagnosed with breast cancer within 2 years of parity might have had a delay in diagnosis as a result of pregnancy or lactation or have delayed

Table 3 Multivariate Cox proportional hazards survival models based on the time since last childbirth among patients with breast cancer

Factors	Status	Hazard ratio	95 % CI	Wald *p* value	3 test *p* value
AJCC stage	Stage 0–1	1			<0.0001
	Stage 2	2.63	1.10–6.30	0.0303	
	Stage 3–4	10.48	4.30–25.55	<0.0001	
Histological grade	Grade 1–2	1			NA
	Grade 3	2.49	1.47–4.21	0.0007	
ER status	Negative	1			NA
	Positive	0.66	0.39–1.12	0.125	
PgR status	Negative	1			NA
	Positive	0.94	0.55–1.60	0.8155	
HER2 status	Negative	1			NA
	Positive	1.08	0.61–1.92	0.7836	
Since last childbirth	Group N	1			0.0695
	Group A	2.19	1.05–4.56	0.0364	
	Group B	1.49	0.79–2.83	0.2231	
	Group C	0.81	0.46–1.43	0.4711	

Adjusted for age at diagnosis, AJCC stage, histological grade, and ER, PgR, and HER2 statuses

NA not applicable, *AJCC* American Joint Committee on Cancer, *HER2* human EGFR-related 2

Table 4 Hazard ratio for death based on the time since last childbirth

Since last childbirth	Unadjusted		Adjusted 1		Adjusted 2	
	HR (95 % CI)	*p*	HR (95 % CI)	*p*	HR (95 % CI)	*p*
Group N	1		1		1	
Group A	3.25 (1.81–5.85)	<0.001	2.26 (1.11–4.59)	0.024	2.19 (1.05–4.56)	0.036
Group B	1.59 (0.86–2.94)	0.141	1.50 (0.79–2.85)	0.210	1.49 (0.79–2.83)	0.223
Group C	0.79 (0.47–1.33)	0.377	0.81 (0.46–1.42)	0.460	0.81 (0.46–1.43)	0.471

Adjusted 1 HR adjusted for AJCC clinical stage (0–1, 2, 3–4), histological tumor grade (1–2, 3), and estrogen receptor status (positive, negative)

Adjusted 2 HR adjusted for age at diagnosis, AJCC clinical stage (0–1, 2, 3–4), histological tumor grade (1–2, 3), estrogen and progesterone receptor status (positive, negative), and HER2 status (positive and negative)

HR hazard ratio, *CI* confidence interval

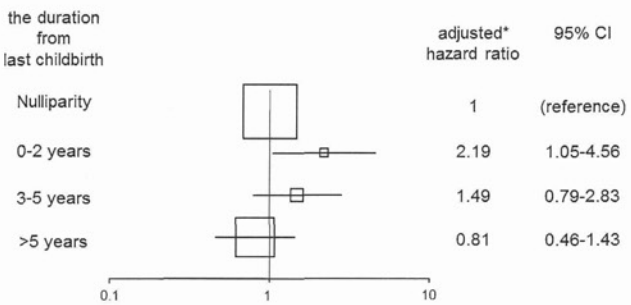

Fig. 2 Multivariate model of mortality based on the time since last childbirth. *Adjusted for age at diagnosis; AJCC clinical stage; histological tumor grade; and ER, PgR, and HER2 statuses; *CI* confidence interval

initiation of therapy until after delivery. Several studies had described that these factors also might have played a role in having an adverse outcome compared with those who had delivered more than 2 years earlier or were nulliparous at diagnosis [18, 21, 23–25]. This study showed that breast cancer patients who delivered within 2 years at diagnosis had more advanced T stage, more regional lymph node metastasis, and higher histological tumor grade compared with those who delivered 3 years or more at diagnosis by trend test. However, the time since last childbirth demonstrated an independent prognostic factor adjusted to tumor characteristics in our study.

The present study was concordant with previous studies showing that breast cancer patients with recent parity tend to have more advanced stage tumors, hormone-receptor negativity, aggressive growth, and high tumor grade, suggesting that pregnancy could have influenced tumor biology [21, 23, 27, 28]. Young breast cancer patients, those included women with recent childbirth, also had more aggressive tumor characteristics, less luminal A tumor, and more TNBC tumor [5, 16, 29, 30]. The present study was also concordant with epidemiological studies showing that recent parity before breast cancer diagnosis is associated with a worse outcome in premenopausal women (generally younger than 45 years), with a peak in risk of death within 2 years after delivery [21–26, 31]. Tumors found in women who have given birth recently have been reported to present with more adverse characteristics compared with tumors in nulliparous women [23, 32]. However, our results revealed that among patients with HR+HER2− tumors, which generally have a good prognosis, women who had given birth recently had a poorer prognosis than nulliparous women, although the reason for recent parity being associated with poor survival has not yet been clearly elucidated.

Pregnancy has a dual effect on the risk of breast cancer. A full-term pregnancy protects against the development of breast cancer later in life because full-term pregnancy induces differentiation of the mammary gland during pregnancy, making it less susceptible to carcinogenic insults [33]. However, shortly after pregnancy the risk of breast cancer increases temporarily, with a peak in risk 5–7 years after delivery [34, 35]. This short-term increase in risk may be because of stimulation of normal mammary gland growth by pregnancy hormones as well as, already existing mammary tumor cells.

Several hypotheses have been proposed to explain the poor prognosis of young breast cancer patients who have recently given birth. Gestational hormones, which are estrogen, progesterone, and insulin-like growth factor, increase tumor cell proliferation [36–40]. Special hormonal environment of pregnancy may influence the biology of more aggressive tumor type. Russo et al. [33] proposed that pregnancy induced differentiation of the mammary progenitor stem cell 1 to stem cell 2, which is less vulnerable to transformation by carcinogenic insult than progenitor stem cell 1. Recently, several studies have shown that the first full-term pregnancy induces a specific genomic signature in breast epithelium [41–43]. In the premenopausal parous human breast, inflammation-associated genes were upregulated and expression of hormone receptor and HER2 was changed compared to the nulliparous human breast of

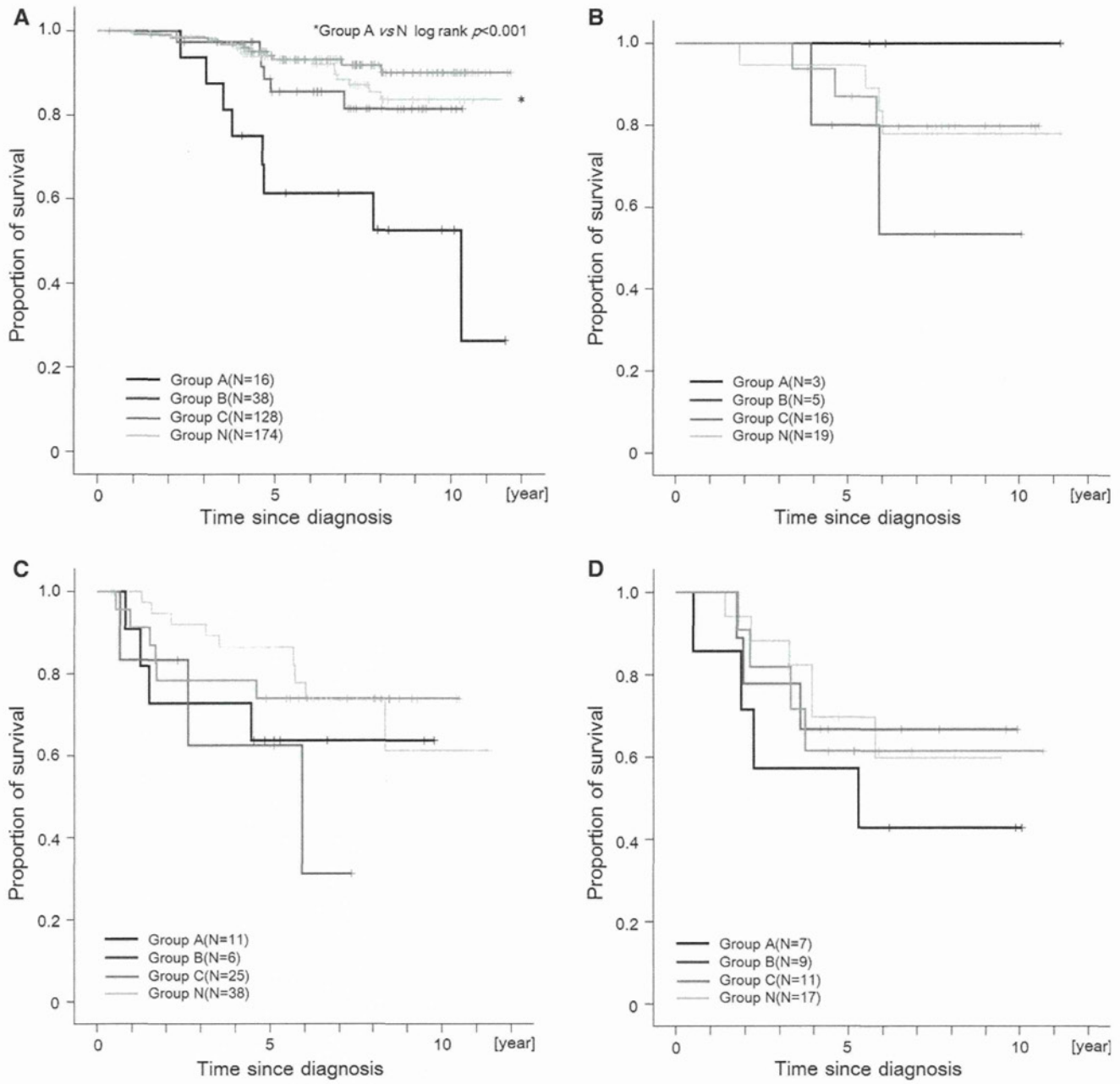

Fig. 3 Kaplan–Meier curves for overall survival according to tumor subtypes. **a** HR+HER2− subtype, **b** HR+HER2+ subtype, **c** HR−HER2− subtype, **d** HR−HER2+ subtype; *HR* hormone receptor

the same generation [42]. The genomic profile of the breast cancer cases, irrespective of parity history, differed from those of parous or nulliparous cancer-free cases according to the hierarchical clustering [41]. This finding suggests that the breast cancer cell was already generated before pregnancy and that pregnancy has contributed to prevention of mammary carcinogenesis. If a breast cancer cell had already been generated before the start of pregnancy, then estrogen and progesterone would mainly promote the proliferation of hormone-receptor-positive breast cancer cells, not negative cells.

This hypothesis cannot explain how a shorter length of time since the last childbirth leads to an increased development of hormone-receptor-negative breast cancer in young breast cancer patients. However, researchers have shown that receptor activator of nuclear factor-κB ligand secreted by progesterone-receptor-expressing epithelial cells stimulated by progesterone induced not only an epithelial proliferative response, but also epithelial carcinogenesis [44, 45]. In addition, RANKL PgR+ differentiated mammary cells stimulated by progesterone, promoted proliferation of the hormone-receptor-negative mammary

progenitor cells. Conversely, Schedin [35] proposed that the period between last childbirth to breast cancer diagnosis involved the process of mammary gland involution, which might facilitate breast cancer metastasis and increase the risk of death. In support of this hypothesis, others have shown that breast cancer patients with recent parity have a higher risk of distant recurrence than nulliparous women [46]. However, our data are not able to provide any proof for above-mentioned hypotheses underlying development of aggressive phenotype in women with recent parity.

Here, we have provided evidence that recent parity is associated with more aggressive histopathological tumor features and worse survival outcomes in breast cancer patients; however, our study does have some limitations. Firstly, since we used an initial routine questionnaire to assess reproductive status, some data was missing from our analysis. In fact, only 85 % of the data regarding breast-feeding status was obtained, although parity data from almost all patients was included in the analysis. Secondly, the questionnaire inquired information about prior use of any hormonal agents including those used for fertility treatment, contraception, and treatment for osteoporosis, but not all patients filled in the form and also their response had not been routinely validated through interview by healthcare providers. Thirdly, although the frequency of *BRCA1/2* germline mutation in Japanese women has been reported to be similar to caucasian in a small study [47], genetic counseling and testing has not been routinely recommended in clinical practice except for selected patients with a strong family history. Moreover *BRCA1/2* testing is not supported by public health insurance. Therefore, only a limited number of patients were offered genetic counseling and testing in this cohort, which disallows analyses according to *BRCA1/2* mutation status. However, family history was neither associated with clinical feature nor prognosis in our cohort (data not shown). Finally, it was not clear whether tumor(s) with poor outcome affected the advanced tumor characteristics or whether the advanced tumor characteristics caused the poor outcomes. However, our findings that breast cancer patients who gave birth more recently had poor outcomes even after adjusting well-known prognostic factors indicate that undiscovered factors associated with recent childbirth induce a change in the mammary glands. Further studies are needed to elucidate the underlying biology.

In conclusion, our results demonstrate that breast cancer patients who had given birth more recently had tumors with more aggressive features and a worse prognosis than patients who were nulliparous or had given birth less recently.

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Conflict of interest The authors declare that they have no conflict of interest.

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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/

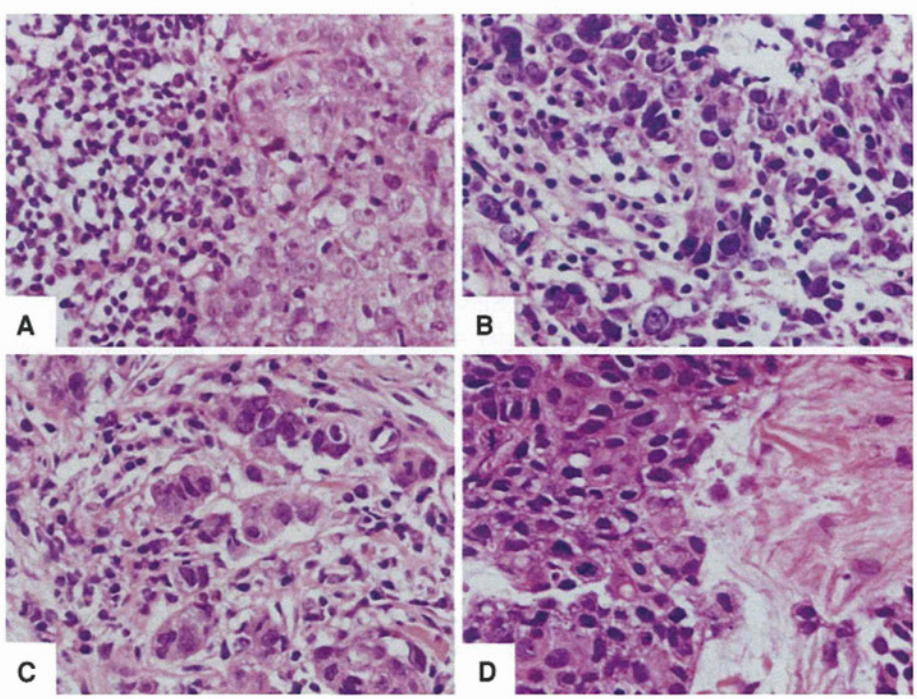

Fig. 1 Histophathological features of tumor-infiltrating lymphocytes (TILs). **a** High TIL score (proportional score 3+ intensity score 2); **b** High TIL score (proportional score 2+ intensity score 2); **c** Low TIL score (proportional score 1+ intensity score 2); **d** Low TIL score (proportional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

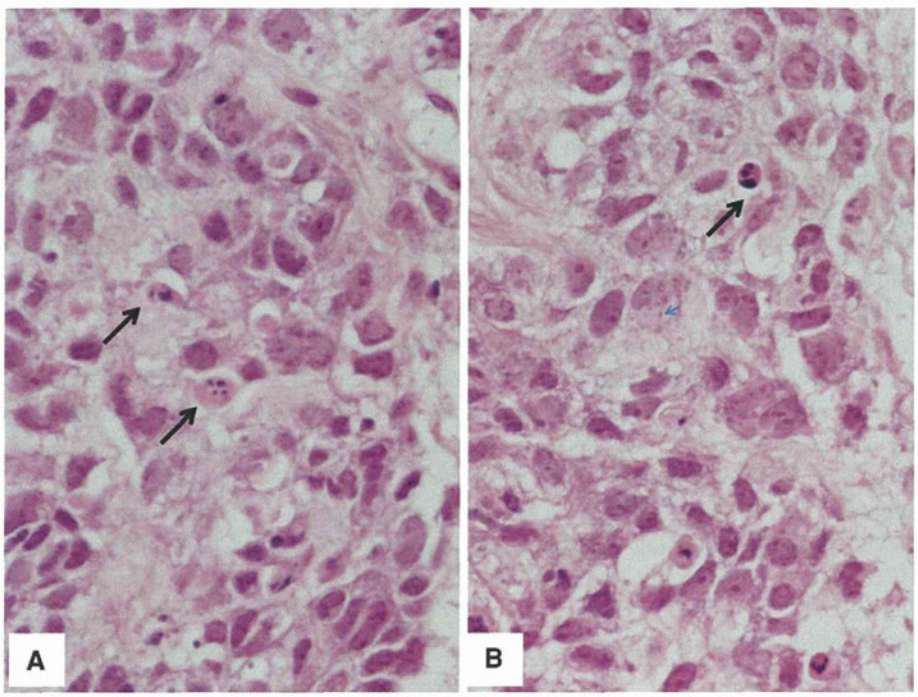

Fig. 2 Histophathological features of breast carcinoma with apoptosis (**a**, **b**) (*arrows*: apoptosis) Original magnification: 400×

6 > 1%, CK14 > 1%, or EGFR > 1%. For reference, data based on the criteria CK5/6 > 10%, CK14 > 10%, or EGFR > 10% were also acquired. p53 was scored using the Allred score and was regarded as positive when ≥5.

Statistical analyses

Statistical analyses were performed using SPSS software. Patients' characteristics were compared between subgroups using the chi-squared test or Fisher's exact test for categorical variables, and Kruskal–Wallis test for continuous variables. Association of pathological parameters, including a basal-like subtype, with pCR, QpCR, or pCR and no residual axillary tumor were evaluated using the chi-squared test or Fisher's exact test. Predictive ratio of pCR, QpCR, or pCR plus residual axillary metastasis by clinicopathological parameters were analyzed using the univariate and multivariate logistic regression models. Survival curves of patients were drawn using Kaplan–Meier method, and statistical difference between survival curves were calculated by using the log-rank test. In all analyses, differences were considered significant at $P < 0.05$.

Results

We confirmed immunohistochemically that all 92 tumors were TNBC, 42 of 50 were of the HR−/HER2+ subtype, and 46 of 50 were of the HR+/HER2− subtype. A total of 180 specimens were investigated in this study. The characteristics of the patients are presented in Tables 1 and 2.

Clinicopathological characteristics and subtypes

In tumors with the TNBC and HR−/HER2+ subtype, the frequencies of the basal-like subtype were 59% (54 of 92) and 43% (18 of 42), respectively, compared with only 7% (3 of 46) in the HR+/HER2− subtype. Therefore, the incidence of the basal-like subtype was significantly higher in TNBC or in the HR−/HER2+ subtype than in the HR+/HER2− subtype ($P < 0.001$). Similarly, the frequency of p53 expression was significantly higher in TNBC (63%, 58 of 92) and the HR−/HER2+ subtype (62%, 26 of 42) than in the HR+/HER2− subtype (26%, 12 of 46) ($P < 0.001$). Tumors of histological grade 3 were more frequent in TNBC (89%, 82 of 92) and the HR−/HER2+ subtype (81%, 34 of 42) than in the HR+/HER2− subtype (13%, 6 of 46) ($P < 0.001$).

The incidence of high TIL score (score 3–5) was also higher in TNBC (73%, 67 of 92) and the HR−/HER2+ subtype (55%, 23 of 42) than in the HR+/HER2− subtype (17%, 8 of 46) ($P = 0.002$). An apoptosis score of 2 was also more frequent in TNBC (21%, 19 of 92) and the HR−/HER2+ subtype (48%, 20 of 42) than in the HR+/HER2− subtype (2%, 1 of 46) ($P < 0.001$). The incidences of a basal-like subtype, p53 expression, a high TIL score, and an apoptosis score of 2 did not differ between TNBC and the HR−/HER2+ subtype.

All six metaplastic carcinomas were TNBC [17].

Table 1 Evaluation of clinicopathological parameters in three subtypes of primary breast cancer

	TNBC ($n = 92$) No. of patients (%)	HR−/HER2+ ($n = 42$) No. of patients (%)	HR+/HER2− ($n = 46$) No. of patients (%)	P value
Age				
Median (range)	52 (23-76)	55 (31-71)	55 (31-71)	0.36
T				
1	2 (2)	0 (0)	0 (0)	0.37
2	48 (53)	17 (41)	26 (56)	
3	27 (29)	16 (38)	11 (24)	
4	15 (16)	9 (21)	9 (20)	
N				
0	45 (49)	24 (57)	24 (52)	0.96
1	35 (38)	14 (33)	18 (39)	
2	10 (11)	3 (7)	3 (7)	
3	2 (2)	1 (3)	1 (2)	
Stage				
II	56 (61)	25 (60)	28 (61)	0.99
III	36 (39)	17 (40)	18 (39)	
ER				
Positive	0 (0)	0 (0)	46 (100)	
Negative	92 (100)	42 (100)	0 (0)	
PgR				
Positive	0 (0)	0 (0)	32 (70)	
Negative	92 (100)	42 (100)	14 (30)	
HER2				
Positive	0 (0)	42 (100)	46 (0)	
Negative	92 (100)	0 (0)	0 (100)	
Basal marker				
Positive	54 (59)	18 (43)	3 (7)	<0.001
Negative	38 (41)	24 (57)	43 (93)	
p53				
Positive	58 (63)	26 (62)	12 (26)	<0.001
Negative	34 (37)	16 (38)	34 (74)	
Grade				
1	1 (1)	0 (0)	4 (9)	<0.001
2	9 (10)	8 (19)	36 (78)	
3	82 (89)	34 (81)	6 (13)	
TIL				
Low (0/1/2)	25 (4/8/13) (27)	19 (7/6/6) (45)	38 (25/8/5) (83)	0.002
High (3/4/5)	67 (22/24/21) (73)	23 (8/11/4) (55)	8 (6/2/0) (17)	
Apoptosis				
0	22 (24)	8 (19)	29 (63)	<0.001
1	51 (55)	14 (33)	16 (35)	
2	19 (21)	20 (48)	1 (2)	
pCR (NSABP B-18)				
Yes	29 (32)	9 (21)	3 (7)	0.004
No	63 (68)	33 (79)	43 (93)	
QpCR (JBCRG 01)				
Yes	35 (38)	17 (40)	3 (7)	<0.001
No	57 (62)	25 (60)	43 (93)	
pCR (primary and lymph nodes)				
Yes	26 (28)	6 (14)	3 (7)	0.006
No	66 (72)	36 (86)	43 (93)	

ER estrogen receptor, *HR* hormone receptors, *pCR* pathological complete response, *PgR* progesterone receptor, *TIL* tumor infiltrating lymphocytes, *TNBC* triple negative breast cancer

Table 2 Correlation between therapeutic effect of primary breast cancer to neoadjuvant chemotherapy (NAC) and infiltrating lymphocytes (TIL)

Subtype of breast cancer and response to NAC	No. of patients (%)			*P*
	Total	TIL score		
		0–2	3–5	
A. TNBC				
pCR (NSABP B-18)				
Yes	29 (32)	4 (16)	25 (37)	0.05
No	63 (68)	21 (84)	42 (63)	
QpCR (JBCRG)				
Yes	35 (38)	4 (16)	31 (46)	0.008
No	57 (62)	21 (84)	36 (54)	
pCR (primary + lymph nodes)				
Yes	26 (28)	4 (16)	22 (33)	0.11
No	66 (72)	21 (84)	45 (67)	
B. HR−/HER2+ subtype				
pCR (NSABP B-18)				
Yes	9 (21)	2 (11)	7 (30)	0.12
No	33 (79)	17 (89)	16 (70)	
QpCR (JBCRG)				
Yes	17 (40)	5 (26)	12 (52)	0.09
No	25 (60)	14 (74)	11 (48)	
pCR (primary + lymph nodes)				
Yes	6 (14)	1 (5)	5 (22)	0.13
No	36 (86)	18 (95)	18 (78)	
C. HR+/HER2− subtype				
pCR (NSABP B-18)				
Yes	3 (7)	2 (5)	1 (13)	0.44
No	43 (93)	36 (95)	7 (87)	
QpCR (JBCRG)				
Yes	3 (7)	2 (5)	1 (13)	0.44
No	43 (93)	36 (95)	7 (87)	
pCR (primary + lymph nodes)				
Yes	3 (7)	2 (5)	1 (13)	0.44
No	43 (93)	36 (95)	7 (87)	
D. Total (TNBC+ HR−/HER2+ HR+/HER2−)				
pCR (NSABP B-18)				
Yes	41 (23)	8 (10)	33 (34)	0.0001
No	139 (77)	74 (90)	65 (66)	
QpCR (JBCRG)				
Yes	55 (31)	11 (13)	44 (45)	< 0.0001
No	125 (69)	71 (87)	54 (55)	
pCR (primary + lymph nodes)				
Yes	35 (19)	7 (9)	28 (29)	0.0007
No	145 (81)	75 (91)	70 (71)	

HR hormone receptors, *TNBC* triple-negative breast cancer, *TIL* tumor-infiltrating lymphocyte, *pCR* pathologically complete response, *QpCR* quasi-pCR, *NAC* neoadjuvant chemotherapy

Clinicopathological characteristics and pCR

The pCR rate according to NSABP B-18 classification was significantly higher in TNBC (32%) and HR−/HER2+ subtype (21%) than in HR+/HER2− subtype (7%) ($P = 0.004$). Likewise, the QpCR rate according to JBCRG 01 classification was significantly higher in TNBC (38%) and HR−/HER2+ subtype (40%) than in HR+/HER2− subtype (7%) ($P < 0.001$). Furthermore, the rate of pCR in both primary site and lymph nodes was significantly higher in TNBC (28%) than in HR−/HER2+ (14%) and HR+/HER2− (7%) subtypes ($P = 0.006$) (Table 1).

Table 3 Correlation between apoptosis of tumor cells and therapeutic effect of primary breast cancer to neoadjuvant chemotherapy (NAC)

Subtype of breast cancer and response to NAC	No. of patients (%)			*P*
	Total	Apoptosis		
		Score 0, 1	Score 2	
A. TNBC				
pCR (NSABP B-18)				
Yes	29 (32)	20 (27)	9 (47)	0.10
No	63 (68)	53 (73)	10 (53)	
QpCR (JBCRG)				
Yes	35 (38)	26 (36)	9 (47)	0.35
No	57 (62)	47 (64)	10 (53)	
pCR (primary + lymph nodes)				
Yes	26 (28)	17 (23)	9 (47)	0.04
No	66 (72)	56 (77)	10 (53)	
B. HR−/HER2+ subtype				
pCR (NSABP B-18)				
Yes	9 (21)	4 (18)	5 (25)	0.71
No	33 (79)	18 (82)	15 (75)	
QpCR (JBCRG)				
Yes	17 (40)	7 (32)	10 (50)	0.23
No	25 (60)	15 (68)	10 (50)	
pCR (primary + lymph nodes)				
Yes	6 (14)	2 (9)	4 (20)	0.40
No	36 (86)	20 (91)	16 (80)	
C. HR+/HER2− subtype				
pCR (NSABP B-18)				
Yes	3 (7)	3 (7)	0 (0)	1.00
No	43 (93)	42 (93)	1 (100)	
QpCR (JBCRG)				
Yes	3 (7)	3 (7)	0 (0)	1.00
No	43 (93)	42 (93)	1 (100)	
pCR (primary + lymph nodes)				
Yes	3 (7)	3 (7)	0 (0)	1.00
No	43 (93)	42 (93)	1 (100)	
D. Total (TNBC+ HR−/HER2+ HR+/HER2−)				
pCR (NSABP B-18)				
Yes	41 (23)	27 (19)	14 (35)	0.04
No	139 (77)	113 (81)	26 (65)	
QpCR (JBCRG)				
Yes	55 (31)	36 (26)	19 (47)	0.008
No	125 (69)	104 (74)	21 (53)	
pCR (primary + lymph nodes)				
Yes	35 (19)	22 (16)	13 (32)	0.02
No	145 (81)	118 (84)	27 (68)	

HR hormone receptors, *TNBC* triple-negative breast cancer, *pCR* pathologically complete response, *QpCR* quasi-pCR, *NAC* neoadjuvant chemotherapy

The association between pCR and TIL scores stratified by tumor subtype is shown in Table 2. In patients with TNBC, the pCR rate was significantly higher in those with tumors showing high TIL scores (3–5) (37%, 25 of 67) than in those with tumor showing low TIL scores (0–2) (16%, 4 of 25) ($P = 0.05$). Likewise, the QpCR rate was significantly higher in those with tumors showing the high TIL scores (46%, 31 of 67) than in those with the low TIL scores (16%, 4 of 25, $P = 0.008$). Furthermore, the rate of pCR in both primary tumor and axillary lymph nodes tended to be higher in the patients with tumors showing the high TIL scores (35%, 22 of 67) than in those with tumors

showing the low TIL scores (16%, 4 of 25). A similar tendency of correlation was seen for tumors of HR−/HER2+ subtype (Table 2), although there was no statistic significance. There was no correlation between TIL and therapeutic effect in HR+/HER2− subtype tumors. In a total of 180 cases including all TNBC, HR−/HER2+, and HR+/HER2− subtypes studied, TIL was significantly correlated with pCR, QpCR, and the pCR in both the primary site and lymph nodes ($P = 0.0001$, $P < 0.0001$, and $P = 0.0007$, respectively, Table 2).

In the patients with TNBC, the pCR rate tended to be higher in those with tumors showing an apoptosis score of 2 (47%, 9 of 19) than in those with an apoptosis score 0 or 1 (27%, 20 of 73, $P = 0.10$) (Table 3). Furthermore, the rate of pCR in both primary tumor and axillary nodes was significantly higher in the tumors showing an apoptosis score 2 (47%, 9 of 19) than in those with an apoptosis score 0 or 1 (23%, 17 of 73, $P = 0.04$). A similar tendency of correlation was seen for tumors of HR−/HER2+ subtype (Table 3), although there was no statistic significance between an apoptosis score and these pCRs (Table 3). There was no statistically significant correlation between apoptosis score and therapeutic effect in HR+/HER2− subtype tumors. In a total of 180 cases including these three subtypes, apoptosis was significantly correlated with pCR, QpCR, and the pCR in both the primary site and axillary lymph nodes ($P = 0.04$, 0.008, and 0.02, respectively) (Table 3).

The pCR rate did not differ significantly between p53-negative tumors (13 of 34, 38%) and p53-positive tumors (15 of 57, 26%) in patients with TNBC. In the HR−/HER2+ subtype, however, seven of nine patients who achieved pCR had p53-positive tumors. There was no correlation between pCR and p53 in the HR+/HER2− subtype.

The pCR rate did not differ between patients with tumors of the basal-like subtype and those with tumors of the non-basal-like subtype (Table 4). Same tendencies of relationship with p53 status or with basal-like subtype were seen for the classification of QpCR and for the pCR of both the primary site and axillary lymph nodes (data not shown).

When all 180 cases were combined, T, N, and grade were correlated or tended to be correlated with pCR (Table 4). QpCR, and the pCR of both primary site and axillary lymph nodes also showed similar tendency (data not shown). Age was not correlated with therapeutic effect.

A univariate regression model analysis showed that the high TIL score was significantly correlated with QpCR (relative ratio (RR) 4.52, 95% reliable range (95%RR) 1.40–14.59) and nearly significantly correlated with pCR in

Table 4 Correlation of clinicopathological parameters with pathological complete response (pCR) of primary breast cancer to neoadjuvant chemotherapy

	All	No. of pCR/No. of patients (%)						
		P value	TNBC	*P* value	HR−/HER2+	*P* value	HR+/HER2−	*P* value
Age								
≤50	14/64 (22)	0.80	11/40 (28)	0.46	3/12 (25)	0.72	0/12 (0)	0.39
>50	27/116 (23)		18/52 (35)		6/30 (20)		3/34 (9)	
T								
1, 2	26/93 (28)	0.09	18/50 (36)	0.31	6/17 (35)	0.07	2/26 (8)	0.60
3, 4	15/87 (17)		11/42 (26)		3/25 (12)		1/20 (5)	
N								
Positive	14/87 (16)	0.03	11/47 (23)	0.09	2/18 (11)	0.15	1/22 (5)	0.53
Negative	27/93 (29)		18/45 (40)		7/24 (29)		2/24 (8)	
Stage								
II	31/109 (28)	0.03	21/56 (38)	0.12	8/25 (32)	0.05	2/28 (7)	0.66
III	10/71 (14)		8/36 (22)		1/17 (6)		1/18 (6)	
Grade								
1, 2	7/58 (12)	0.02	3/10 (30)	0.91	1/8 (13)	0.44	3/40 (8)	0.65
3	34/122 (29)		26/82 (32)		8/34 (24)		0/6 (0)	
Basal-like								
Positive	23/75 (31)	0.03	19/54 (35)	0.36	4/18 (22)	0.60	0/3 (0)	0.81
Negative	18/105 (17)		10/38 (26)		5/24 (21)		3/43 (7)	
p53								
Positive	23/95 (24)	0.52	15/57 (26)	0.23	7/26 (27)	0.24	1/12 (8)	0.61
Negative	17/84 (20)		13/34 (38)		2/16 (13)		2/34 (6)	

HR hormone receptors, *pCR* pathological complete response

Table 5 Logistic analysis for prediction of pathological therapeutic effect to neoadjuvant chemotherapy to TNBC

	Relative ratio (95% reliable range)	*P* value
A. Univariate		
1. pCR (NSABP B-18)		
TIL (score 3-5 vs. 0-2)	3.12 (0.96–10.15)	0.058
Apoptosis (2 vs. 0, 1)	2.38 (0.85–6.73)	0.10
2. QpCR (JBCRG)		
TIL (score 3-5 vs. 0-2)	4.52 (1.40–14.59)	0.012
Apoptosis (2 vs. 0, 1)	1.63 (0.59–4.51)	0.35
3. pCR (primary + lymph node)		
TIL (score 3-5 vs. 0-2)	2.57 (0.79–8.39)	0.12
Apoptosis (2 vs. 0, 1)	2.97 (1.04–8.49)	0.043
B. Multivariate		
1. pCR (NSABP B-18)		
TIL (score 3-5 vs. 0-2)	2.78 (0.84–9.18)	0.09
Apoptosis (2 vs. 0, 1)	2.01 (0.70–5.81)	0.20
2. QpCR (JBCRG)		
TIL (score 3-5 vs. 0-2)	4.34 (1.33–14.21)	0.015
Apoptosis (2 vs. 0, 1)	1.27 (0.44–3.65)	0.66
3. pCR (primary + lymph node)		
TIL (score 3-5 vs. 0-2)	2.17 (0.65–7.28)	0.21
Apoptosis (2 vs. 0, 1)	2.60 (0.89–7.58)	0.08

pCR pathological complete response, *TIL* tumor-infiltrating lymphocyte, *TNBC* triple-negative breast cancer

N, *T*, grade, basal-like, p53, and histological type were not significant as predictor of pCR

92 TNBCs (relative ratio 3.12, 95%RR 0.96–10.15) ($P = 0.012$ and 0.058, respectively) (Table 5). Apoptosis was significantly correlated with pCR (primary + lymph node) in 92 TNBCs (RR 2.97, 95%RR 1.04–8.49) ($P = 0.043$). Other parameters, including T, N, grade, basal-like subtype, p53 and histological type, were not significant predictors of pCR. TIL and apoptosis showed no mutual correlation. When these two parameters were subjected to multivariate analysis, only TIL was shown to be a significant independent factor for QpCR (RR 4.34, 95%RR 1.33–14.21, $P = 0.015$), but apoptosis was not significant (Table 5).

Survival analyses

In 92 patients with TNBC, disease-free survival (DFS) curves differed significantly between pCR and non-pCR groups (5-year DFS rate 93% vs. 66%, $P = 0.019$), between QpCR and non-QpCR groups (5-year DFS rate 91% vs. 64%, $P = 0.010$), and between the group of pCR in both primary tumor and axillary lymph nodes and others (5-year DFS rate 92% vs. 68%, $P = 0.043$) (Fig. 3). In TNBC, patients with a high TIL score tumor showed slightly higher 5-year DFS rate than patients with a low TIL score tumor (77% vs. 70%), but the difference was not significant statistically ($P = 0.58$) (Fig. 4).

Discussion

Breast cancer has been shown to be a heterogeneous disease, and each intrinsic subtype of breast cancer differs in terms of gene expression and molecular features [1–5]. Previous studies reported differences between breast cancer subtypes in the pCR rate after primary chemotherapy [8, 10]: Rouzier et al. reported that the pCR rate after anthracycline and taxane chemotherapy in patients with luminal subtypes was 6%, while patients with both the basal-like and erbB2+ (HER2) subtypes had a pCR rate of 45%, based on classification using a "breast intrinsic" gene set [8]. Carey et al. also reported differences in the chemosensitivity of breast cancer subtypes when classified by immunohistochemistry: pCR rates after treatment with anthracycline either alone or in combination with taxane were 27, 36, and 7% for TNBC, and the HER2 and luminal subtypes, respectively [10]. In the present study, we confirmed that the pCR rate, QpCR rate, and the pCR rate in both the primary site and lymph nodes were significantly higher in patients with TNBC and tumors of the HR−/HER2+ subtype than in those with tumors of the HR+/HER2− subtype.

The proportions of cases showing a high TIL score (3, 4 or 5) and high apoptosis (score 2) were larger in TNBC and the HR−/HER2+ subtype than in the HR+/HER2− subtype. In addition, both TIL score and apoptosis were significantly associated with a response to NAC in TNBC, while in the HR−/HER2+ subtype and the HR+/HER2− subtype, these parameters were not significantly associated with pCR or QpCR. Because we used statistical tests on multiple related hypotheses, i.e., pCR, QpCR, and pCR in both the primary tumor and axillary lymph nodes, the data acquired should be considered exploratory. Nonetheless, these results suggest that patients with a high immune response to TNBC were more likely to show pCR, and that the immune component played a substantial role in the response of TNBC to NAC.

Although conflicting results have been reported [20, 21], earlier studies revealed a relationship between high lymphocyte infiltration and good prognosis in patients with breast cancer [22–25]. However, breast cancer subtypes were not taken into consideration in these studies. Kreike et al. demonstrated that a large amount of lymphocytic infiltrate was a significant indicator of longer distant metastasis-free survival in patients with TNBC [26]. In several studies, changes in TIL score or in the percentage in a certain subset of T cells were shown to be correlated

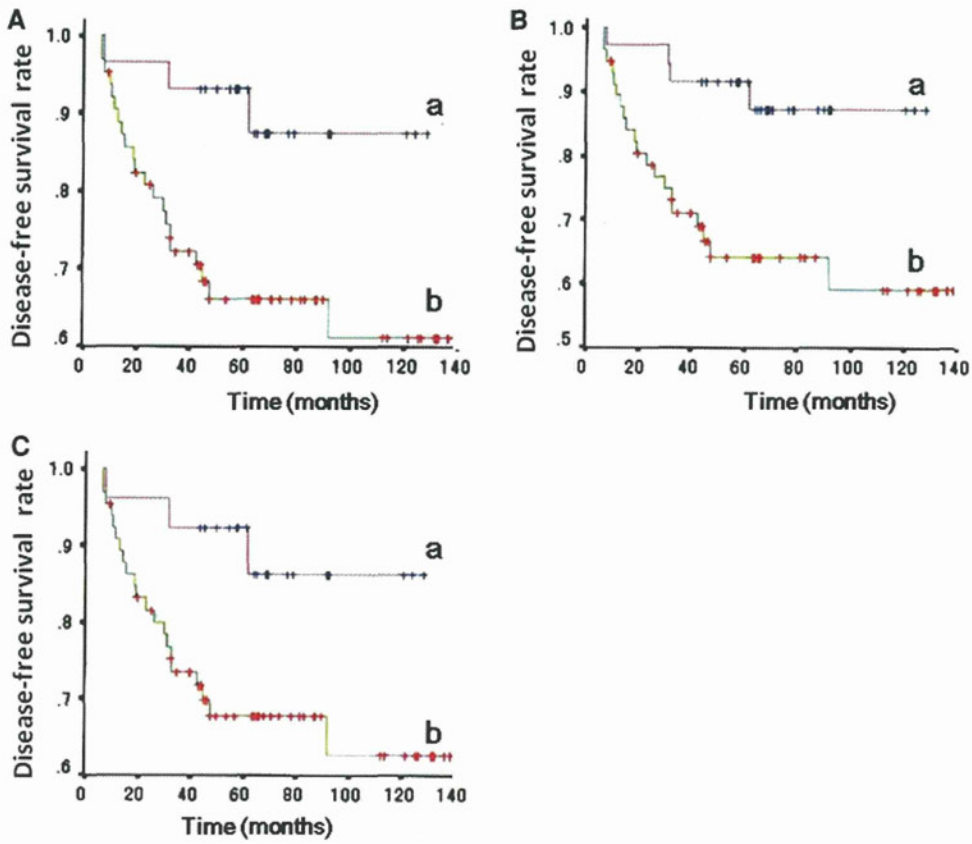

Fig. 3 Disease-free survival curves for patients with primary triple-negative breast cancer (TNBC) after neoadjuvant chemotherapy. **a** Survival curves for (*a*) patient group that showed pCR (NSABP B-18) and (*b*) patient group that showed non-pCR. Curves for two groups are significantly different (5-year DFS rate 93% vs. 66%, $P = 0.019$). **b** Survival curves for (*a*) patient group that showed QpCR (JBCRG) and (*b*) patient group that showed non-QpCR. Curves for two groups are significantly different (5-year DFS rate 91% vs. 64%, $P = 0.010$). **c** Survival curves for (*a*) patient group that showed pCR and (*b*) patient group that showed non-pCR in both primary tumor and axillary lymph nodes and others. *Curves* for two groups are significantly different (5-year DFS rate 92% vs. 68%, $P = 0.043$)

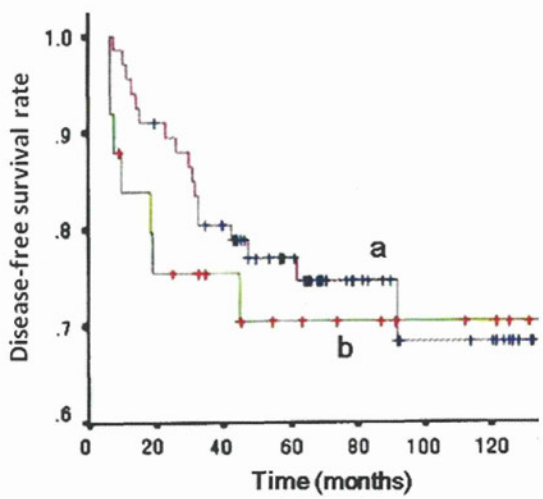

Fig. 4 Disease-free survival curves for patients with primary triple-negative breast cancer (TNBC) after neoadjuvant chemotherapy, stratified by the score of tumor infiltrating lymphocytes (TIL). **a** High TIL score group ($n = 67$). **b** Low TIL score group ($n = 25$). Although the 5-year disease-free survival rate was slightly higher in the high TIL score group (77%) than in the low TIL score group (70%), these two curves did not differ significantly ($P = 0.58$)

with pCR to neoadjuvant chemotherapy of breast cancer [27, 28].

It is also possible that gene expression associated with chemosensitivity and prognosis differs among breast cancer subtypes. Teschendorff et al. also reported that a high level of gene expression representing an immune response was correlated with the better prognosis of patients with ER-negative breast cancer [29]. In fact, Rouzier et al. demonstrated that the genes predictive of pCR differed between the basal-like subtype and the HER2 subtype [8]. Furthermore, Desmedt et al. revealed that the gene expression modules associated with clinical outcome were different between the ER-/HER2− and HER2+ tumors: immune response genes only in the former and both tumor invasion and immune response genes in the latter [5]. Their results were consistent with those of the present study, which demonstrated a significant correlation between the presence of TIL and pCR/QpCR rate in TNBC, but the correlation was only marginal in the HR−/HER2+ subtype. Therefore, the molecular mechanisms determining chemosensitivity may differ between the basal-like and HR−/HER2+ subtypes.

We demonstrated a tendency of correlation between apoptosis and response to NAC in TNBC. Although Desmedt et al. examined the gene expression module associated with apoptosis, there was no association between expression of this gene set and prognosis in any of the breast cancer subtypes examined [5]. Because apoptosis has been defined as programmed cell death, and is usually unaccompanied by inflammation and cytokine release, apoptosis has been believed to be independent of TIL. In