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).

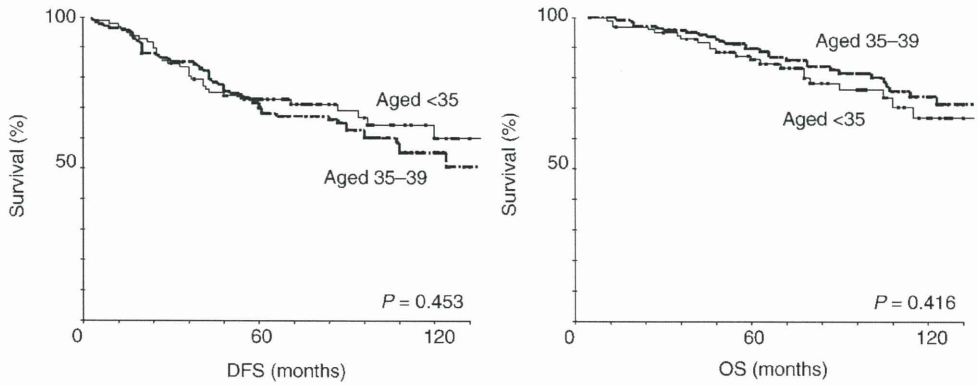
DISCUSSION

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.

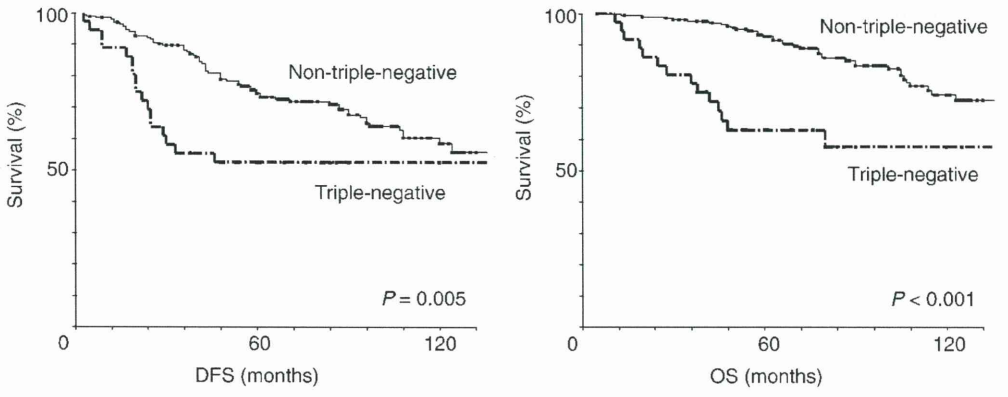
In contrast to our findings, de la Rochefordiere et al. (19) reported that, in a series of 1703 patients from a single institution, the relationship between recurrence hazard and age was best fitted by a log-linear function that indicated a 4% decrease in recurrence and a 2% decrease in death for every 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.

Funding

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Conflict of interest statement

None declared.

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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/

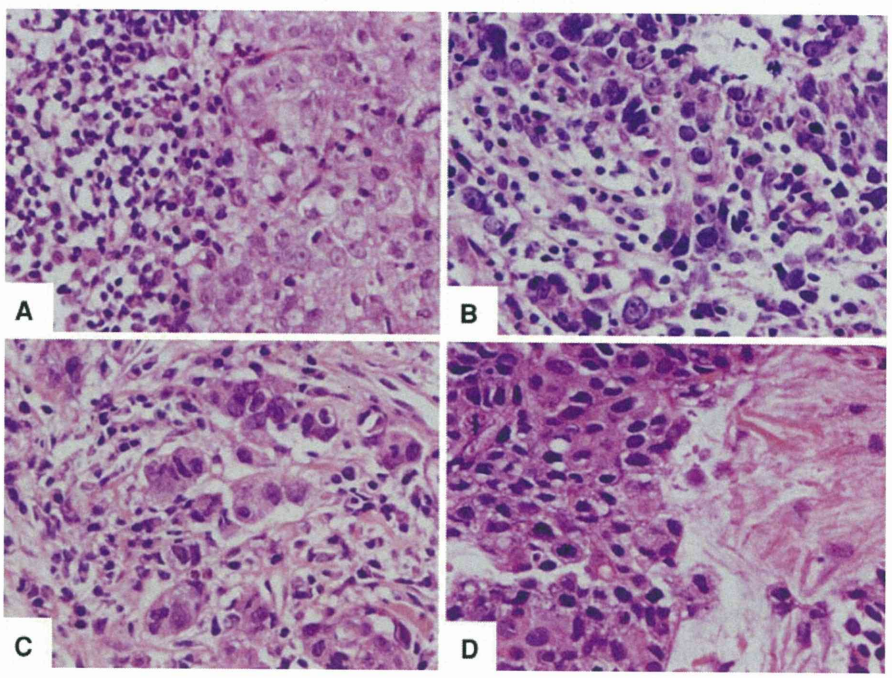

Fig. 1 Histophathological features of tumor-infiltrating lymphocytes (TILs). **a** High TIL score (proportional score 3+ intensity score 2); **b** High TIL score (proportional score 2+ intensity score 2); **c** Low TIL score (proportional score 1+ intensity score 2); **d** Low TIL score (proportional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

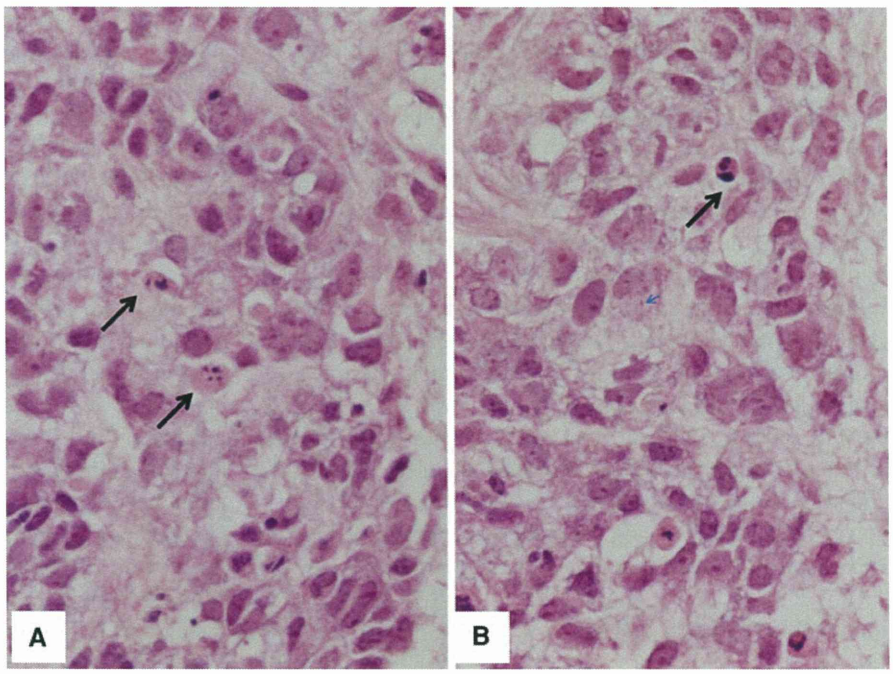

Fig. 2 Histophathological features of breast carcinoma with apoptosis (**a**, **b**) (*arrows*: apoptosis) Original magnification: 400×

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

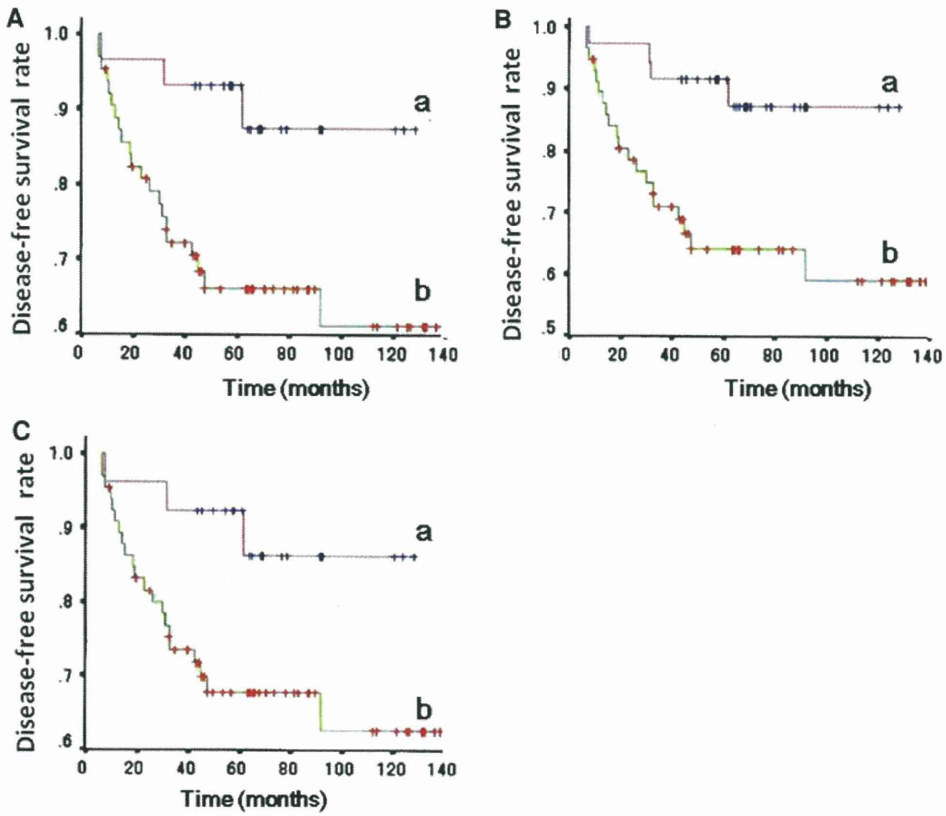

Fig. 3 Disease-free survival curves for patients with primary triple-negative breast cancer (TNBC) after neoadjuvant chemotherapy. **a** Survival curves for (*a*) patient group that showed pCR (NSABP B-18) and (*b*) patient group that showed non-pCR. Curves for two groups are significantly different (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%, $P = 0.043$)

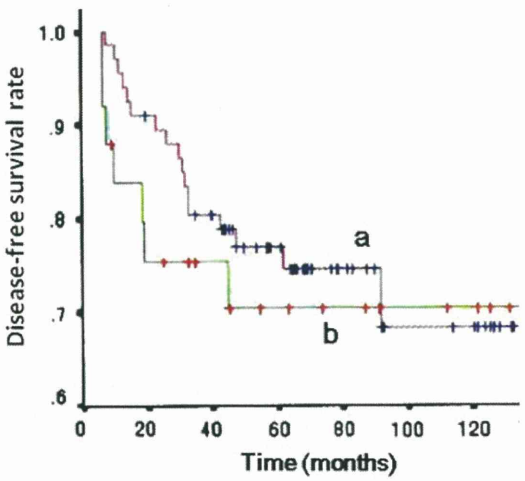

Fig. 4 Disease-free survival curves for patients with primary triple-negative breast cancer (TNBC) after neoadjuvant chemotherapy, stratified by the score of tumor infiltrating lymphocytes (TIL). **a** High TIL score group ($n = 67$). **b** Low TIL score group ($n = 25$). Although the 5-year disease-free 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

the present study, there was no significant relationship between the presence of TIL and tumor cell apoptosis in TNBC. However, recent studies demonstrated that tumor cell death induced by chemotherapy can promote cytotoxic T-lymphocyte response that confers permanent antitumor immunity [30, 31]. We used histological examination only to identify apoptotic cancer cells. However, it would be more informative to add other techniques, such as the TUNEL method or immunohistochemistry, to identify apoptosis from multiple angles.

We revealed no correlation between the expression of basal-like markers and response to NAC in all of the breast subtypes examined. Although the significance of basal-like markers for clinical outcome is controversial [32–34], a lack of association between basal-like markers and chemosensitivity or prognosis has been demonstrated when breast cancers are divided into subtypes on the basis of ER and HER2 positivity [33, 34]. Nuclear p53 has been shown to be frequent in TNBC [35], but the significance of p53 as a predictive marker for pCR is also controversial [36]. In the present study we were unable to demonstrate any significant impact of p53 as such a marker.

It is unknown whether TILs cause susceptibility to chemotherapy, or they are simply a possible marker of chemosensitivity. There are reports that showed TILs are a predictor of response to neoadjuvant chemotherapy in breast cancer [37, 38]. Hornychova et al. reported that the infiltration of $CD3^+$ T-lymphocytes and $CD83^+$ dendritic cells were correlated with the effectiveness of primary chemotherapy, evaluated as pCR [38]. Denkert et al. showed that T-cell-related markers CD3D and CXCL9 expression were significantly associated with pCR [37]. Several studies suggested possible mechanisms of tumor-immune interaction in response to chemotherapy. pCR to neoadjuvant chemotherapy was shown to be associated with an immunologic profile combining the absence of immunosuppressive $Foxp3^+$ regulatory T cells and the presence of a high number of $CD8^+$ T cells and cytotoxic cells [28]. These reports suggest subsets of TILs caused susceptibility to chemotherapy.

In conclusion, we have demonstrated that the various breast cancer subtypes classified by ER, PgR, and HER2 status have different pathological characteristics and predictive factors for response to chemotherapy. TNBC with a high score for TIL and apoptosis is more likely to respond to chemotherapy. Therefore, in patients with TNBC, the immune response appears to influence on the response to chemotherapy. Further examination is warranted to elucidate the mechanism involved in the immune response component of chemosensitivity.

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