In conclusion, the OSNA assay is considered reliable in the clinical setting for the routine intraoperative examination of SLN and is useful because it can be performed easily by a nonpathologist. However, further studies to obtain long-term follow-up data for greater numbers of patients are needed to confirm the clinical significance, especially the prognostic impact, of results of the OSNA assay of SLNB for breast cancer.

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CONFLICT OF INTEREST DISCLOSURES

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Original article

The differences in the histological types of breast cancer and the response to neoadjuvant chemotherapy: The relationship between the outcome and the clinicopathological characteristics

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ABSTRACT

Although effective regimens have been established for invasive ductal carcinoma-not otherwise specified (IDC), the efficacy and prognosis of other minor types of breast cancer are unknown because of their rareness. The clinicopathological features and prognosis of other minor types concerning the response to neoadjuvant chemotherapy (NAC) were evaluated in this study.

A total of 562 patients were classified according to the Japanese and the World Health Organization (WHO) classifications, and the number of IDC and other special types (SP) was 500 and 62. The SP patients had a significantly poorer clinicopathological response to NAC and less breast-conservative therapy than those with IDC. According to the WHO classification, mucinous carcinoma, metaplastic carcinomas and apocrine carcinoma also responded poorly, and patients with metaplastic carcinomas and invasive lobular carcinoma had a significantly poorer prognosis. Despite the poor response to chemotherapy, patients with mucinous carcinoma and apocrine carcinoma had a good prognosis.

The response to NAC and the prognosis vary for each histological type. For some types, the prognosis was not related to the clinicopathological response to NAC.

Background: In the treatment of breast cancer, neoadjuvant chemotherapy (NAC) has become the standard treatment modality for downstaging purposes. Although effective regimens have been established for the treatment of invasive ductal carcinoma-not otherwise specified (IDC), the data about the efficacy and prognosis for patients with other minor types of breast cancer are insufficient because of the rareness of these tumors. Defining the relationship between each histological type and the clinicopathological response to NAC is essential to optimizing individualized treatment.

Methods: We retrospectively evaluated the clinicopathological features and classification of the histological types based on the Japanese and the World Health Organization (WHO) classifications before and after NAC in 562 patients with primary breast cancer who underwent curative treatment after NAC between 1998 and 2008. The prognosis was estimated for each histological type.

Results: Of the 562 patients, the number of cases of IDC and other special types (SP) was 500 and 62. In the SP group, the clinicopathological response to NAC was significantly poorer, and the patients underwent breast-conservative therapy less frequently than did the IDC patients. According to the WHO classification, mucinous carcinoma, metaplastic carcinomas and apocrine carcinoma responded poorly to NAC. The disease-free survival and overall survival were significantly worse for patients with metaplastic carcinomas ($p < 0.001$ and $p < 0.001$) and with invasive lobular carcinoma ($p = 0.03$ and $p < 0.001$) than other cancers. Despite their poor response to treatment, patients with mucinous carcinoma and apocrine carcinoma had a good prognosis.

Conclusions: The response to standardized NAC and prognosis varies for each histological type. For some types, the prognosis was not associated with the clinicopathological response to NAC. Innovative regimens should therefore be investigated for each histological type to achieve the best response.

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Introduction

In the treatment of breast cancer, neoadjuvant chemotherapy (NAC) has become the standard treatment modality for down-staging purposes. With the introduction of NAC, many patients have been able to be treated with breast-conserving therapy (BCT) as a result of the tumor reduction prior to surgery. Especially for patients with invasive ductal carcinoma-not otherwise specified (IDC), NAC had been confirmed to be efficient and beneficial, and is now widely applied for treatment. At present, invasive breast carcinoma is treated with a standardized regimen of NAC, regardless of the pathological type. However, because of their rareness, the efficiency and outcomes of NAC for the other minor types of breast carcinoma have not been fully elucidated.

In this study, we made a comparison between the patients with IDC and other types of breast cancer about clinicopathological features with regard to NAC. The histological types were classified using the Japanese classification[1,2] and the World Health Organization (WHO) classification.[3] We have correlated these histological types with the overall survival (OS) and disease-free survival (DFS) of the patients, and assessed the association between the tumor response to standardized NAC and the outcome for each histological type.

Material and methods

Patients

This study was a retrospective analysis of 562 breast cancer patients who underwent NAC during the period from 1998 to 2008 at the National Cancer Center Hospital, Tokyo, Japan. NAC was indicated for clinical stage II tumors that were larger than 3 cm in diameter, and for all stage III tumors. Axillary lymph node metastasis was diagnosed by cytology or imaging studies. Prior to NAC, all the patients underwent a core needle biopsy (CNB) for histological examination and were staged according the International Union Against Cancer (UICC) TNM classification.

Neoadjuvant chemotherapy regimens

NAC regimens were introduced based on current reviews at the time. Anthracycline-based chemotherapy included four cycles of CEF (cyclophosphamide 500 mg/m^2, epirubicin 100 mg/m^2, and fluorouracil 500 mg/m^2) every 3 weeks, or four cycles of AC (doxorubicin 60 mg/m^2, and cyclophosphamide 600 mg/m^2). Taxane chemotherapy included 12 cycles of weekly paclitaxel (wPTX, 80 mg/m^2).[4,5] Concurrent anthracycline and taxane chemotherapy included four cycles every 3 weeks of doxorubicin and docetaxel (AT, 50 and 60 mg/m^2).[6] Sequential anthracycline and taxane chemotherapy included AT (two cycles) followed by wPTX, AC followed by wPTX, and CEF followed by wPTX. Trastuzumab (first cycle; 4 mg/kg: after second cycle; 2 mg/kg) combined with anthracycline and taxane chemotherapy was administered to the patients with overexpression of the human epidermal growth factor receptor 2 (HER2).[7]

Histological diagnosis and evaluation

Prior to NAC, CNB specimens were examined for the histological sub-type and histological grade (HG) by hematoxylin and eosin (HE) staining. After NAC, the surgical specimen was examined for the histological sub-type, HG, and presence or absence of lymphatic or vascular space invasion. The histological sub-types were defined based on the General Rules for Clinical and Pathological Recording of Breast Cancer that were proposed by The Japanese Breast Cancer Society (JBCS classification)[1,2] and the WHO classification.[3] As the feature of the Japanese histological classification, all breast carcinomas are first classified according to the existence of invasion while, in addition, invasive carcinoma is classified as invasive ductal carcinoma, or other types called 'special types (SP)', and the SP category includes invasive lobular carcinoma (ILC) and other minor histological types.

The HG was assessed using the Scaff-Bloom-Richardson classification.[8] Immunohistochemistry was used to examine the tissue samples for the expression of the estrogen receptor (ER), progesterone receptor (PgR), and HER2. The cutoff values for the ER and PgR were 10% positive cells. HER2 status was defined based on immunohistochemical staining (IHC). The specimens that were HER2 2 + by IHC were then subjected to fluorescence *in situ* hybridization (FISH). HER2 positive samples were defined as those that were HER2 3+ in IHC or HER2 2+ in IHC and had an amplification ratio in FISH of >2.0. The degree of lymphatic invasion (ly) was classified by HE staining as follows: absent, no lymphatic invasion; ly1+, minimal lymphatic invasion; ly2+, moderate lymphatic invasion; and ly3+, marked lymphatic invasion. These diagnoses and evaluations were performed separately by two qualified pathologists, and the final diagnosis and evaluations were decided as a result of conferences between the pathologists.

Evaluation of the response to NAC

Prior to and after NAC, all of the patients and tumors were evaluated by physical examinations and radiographic imaging. The tumor diameter was evaluated using calipers and by ultrasonography. The clinical response was assessed using the Response Evaluation Criteria in Solid Tumor (RECIST) guidelines.[9] The tumor was judged to be 'progressive disease (PD)' when the tumor size increased by 20% or more. At that time, chemotherapy was discontinued and surgery was performed. The pathological response was evaluated from surgical specimens. The histopathological response was assessed using the General Rules for Clinical and Pathological Recording of Breast Cancer.[10] Response grade 0 was no response, and was defined by almost no change in the cancer cells after treatment. Grade 3 was a complete response, and was defined as necrosis or the disappearance of all tumor cells. The definition of a pathological complete response (pCR) was 'necrosis and the disappearance of all invasive cells' of the primary tumor. Cases with only intraductal carcinoma remaining were included in the pCR category.

Table 1
The Japanese histological classification of breast tumors (extraction) and the number of patients with each histological type (n = 562).

Histological type	No. of patients	%
B. Malignant (Carcinoma)		
a. Invasive carcinoma	500	89.0
a1. Papillotubular carcinoma	126	22.4
a2. Solid-tubular carcinoma	202	35.9
a3. Scirrhous carcinoma	172	30.6
b. Special types	62	11.0
b1. Mucinous carcinoma	12	2.1
b2. Medullary carcinoma	0	0
b3. Invasive lobular carcinoma	29	5.2
b4. Adenoid cystic carcinoma	0	0
b5. Squamous cell carcinoma	5	0.9
b6. Spindle cell carcinoma	4	0.7
b7. Apocrine carcinoma	5	0.9
b8. Carcinoma with cartilaginous and/or osseous metaplasia	1	0.2
b9. Tubular carcinoma	0	0
b10. Secretory carcinoma	1	0.2
b11. Invasive micropapillary carcinoma	1	0.2
b12. Matrix-producing carcinoma	4	0.7
b13. Others	0	0

Table 2
The administered NAC regimens ($n = 562$).

	No. of patients	%
AT	150	26.7
AT followed by wPTX	25	4.4
AT followed by wPTX/Trastuzumab	2	0.4
AC followed by wPTX	142	25.3
AC followed by wPTX/Trastuzumab	17	3.0
CEF followed by wPTX	181	32.2
CEF followed by wPTX/Trastuzumab	26	4.6
wPTX	12	2.1
wPTX/Trastuzumab	7	1.2

AT, doxorubicin and docetaxel; wPTX, weekly paclitaxel; AC, doxorubicin and cyclophosphamide; CEF, cyclophosphamide, epirubicin and fluorouracil.

Surgery and post-operative treatment

The breast surgery was either a lumpectomy or a total mastectomy. When the patient who underwent a lumpectomy was detected to have cancer in the pathological margin, additional excision was performed until the specimen became pathologically margin free. All of the patients underwent axillary lymph node dissection (level II). Adjuvant therapy was given in some cases based on the most current recommendations from the St. Gallen's Consensus Meeting at the time.[11–15] Tamoxifen (20 mg/day) or anastrozole (1 mg/day) was administered for five years when CNB specimens or surgical postchemotherapy specimens were positive for the ER or PgR. Radiotherapy was performed for the patients who underwent BCT for the residual breast or the patients with tumors >5 cm and/or with massive metastatic lymph nodes (≥4 nodes) for the chest wall, axilla, and supraclavicular area.

Follow-up and statistical analysis

The number of follow-up months was recorded from the first day of NAC to the most recent medical visit on record.

OS and DFS were calculated using the Kaplan–Meier methods and compared using the log-rank test. For comparisons of categorical variables, the chi-square test was used. Odds ratios (OR) and associated 95% confidence intervals (95% CI) were calculated as estimates of the relative risk. Values of $p < 0.05$ were considered to be statistically significant. All data were analyzed using the SPSS software program (SPSS Inc., Chicago, IL).

Results

Patient characteristics and clinical features

Table 1 presents the Japanese histological classification and the number of each histological type. The total number of IDC and SP

Table 3
The results of the analysis of the patient and tumor characteristics by histological groups (JBCS).

	Univariate			Multivariate	
	IDC ($n = 500$)	SP ($n = 62$)	p value	OR (95% CI)	p value
Age, mean ± SD	50.7 ± 10.4	50.6 ± 11.7	0.932		
Age (years)			0.335		
<41	74 (14.8)	13 (21.0)			
41–50	147 (29.4)	15 (24.2)			
51–60	178 (0.356)	18 (29.0)			
≥61	101 (0.202)	16 (25.8)			
Tumor size (cm), mean ± SD					
Prior NAC	5.7 ± 1.7	5.5 ± 2.5	0.075		
After NAC	2.1 ± 1.9	3.5 ± 2.7	<0.001	1.318 (1.063–1.632)	0.012
Stage			0.841		
II	320 (64.0)	39 (62.9)			
III	180 (36.0)	23 (37.1)			
Hormone receptors					
ER positive (%)	223 (44.6)	27(43.5)	0.892		
PgR positive (%)	198 (39.6)	21 (33.9)	0.408		
HER2 positive (%)	105 (21.0)	4 (6.5)	0.006	0.275 (0.080–0.948)	0.041
Histological grade			<0.001	0.674 (0.403–1.125)	0.131
G1 (%)	32 (6.4)	14 (22.6)			
G2 (%)	216 (43.2)	27 (43.5)			
G3 (%)	252 (50.4)	21 (33.9)			
Clinical response					
Responded (CR + PR) (%)	425 (85.0)	42 (67.7)	0.002	0.841 (0.341–2.076)	0.707
CR	165 (33.0)	6 (9.6)	<0.001	0.938 (0.633–1.390)	0.750
PD	13 (2.6)	7 (11.3)	0.003	5.279 (1.715–16.249)	0.004
BCT cases (%)	208 (53.0)	16 (25.8)	0.019	0.386 (0.082–1.247)	0.240
Pathological response					
pCR	113 (22.6)	5 (8.1)	0.080		
Pathological response grade					
G0 (%)	15 (3.0)	6 (9.7)	0.021	2.911 (0.777–10.909)	0.113
G3 (%)	65 (13.0)	5 (8.1)	0.314		
G0/1 (%)	312 (62.4)	42 (67.7)	0.086		
G2/3 (%)	188 (37.6)	20 (32.3)			
Cases of LN metastasis (%)	265 (53.0)	38 (61.3)	0.227		
No. of LN metastasis, mean ± SD	2.8 ± 5.1	3.7 ± 6.6	0.215		
Lymphatic invasion					
present	133 (26.6)	12 (19.4)	0.042	0.385 (0.174–0.851)	0.018
ly(1+)	85 (17.0)	10 (16.1)	0.302		
ly(2+/3+)	48 (9.6)	2 (3.2)	0.018	0.324 (0.073–1.448)	0.140
Vascular invasion, present	17 (3.4)	2 (3.2)	1.000		

IDC, invasive ductal carcinoma-not otherwise specified; SP, special types; OR, odds ratio; CI, confidence interval; SD, standard deviation; NAC, neoadjuvant chemotherapy; ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor receptor 2; G, grade; CR, complete response; PR, partial response; PD, progressive disease; BCT, breast-conserving therapy; pCR, pathological complete response; LN, lymph node.

Table 4
The results of the univariate analysis of the patient and tumor characteristics for each histological type (WHO).

	IDC ($n = 500$)	ILC($n = 29$)		Metaplastic ($n = 14$)		Mucinous ($n = 12$)		Apocrine ($n = 5$)	
			p value		p value		p value		p value
Tumor size (cm), mean + SD									
Prior NAC	5.0 + 1.7	4.7 + 1.2	0.169	5.9 + 2.9	0.254	7.4 + 3.4	0.036	5.3 + 1.4	0.709
After NAC	2.1 + 1.9	2.4 + 1.7	0.400	5.9 + 3.9	0.003	4.1 + 1.7	0.001	3.0 + 0.1	<0.001
Hormone receptors									
ER positive (%)	223 (44.6)	14 (50.0)	0.698	1 (7.1)	0.005	9 (75.0)	0.039	0 (0)	0.061
PgR positive (%)	198 (39.6)	13 (46.4)	0.556	1 (7.1)	0.011	6 (50.0)	0.348	1 (25.0)	0.163
HER2 positive (%)	105 (21.0)	2 (7.1)	0.091	0 (0)	0.085	1 (8.3)	0.252	1 (20.0)	0.999
Histological grade			0.069		0.016		<0.001		0.372
G1	32 (6.4)	4 (14.3)		0 (0)		8 (66.7)		1 (20.0)	
G2	216 (43.2)	17 (60.7)		1 (7.1)		3 (25.0)		4 (80.0)	
G3	252 (50.4)	7 (25.0)		13 (92.9)		1 (8.3)		0 (0)	
Clinical response									
Responded (CR + PR)	425 (85.0)	21 (75.0)	0.211	5 (35.7)	0.003	9 (75.0)	0.271	5 (100)	0.446
CR	165 (33.0)	5 (17.9)	0.067	0 (0)	0.007	0 (0)	0.009	0 (0)	0.137
PD	13 (2.6)	0 (0)	0.428	7 (50.0)	<0.001	0 (0)	0.603	0 (0)	0.737
Pathological response									
pCR	113 (22.6)	2 (7.1)	0.032	0 (0)	0.047	0 (0)	0.045	0 (0)	0.272
Pathological response grade			0.357		0.094		0.116		0.372
G0/1	312 (62.4)	19 (67.9)		12 (85.7)		10 (83.3)		2 (40.0)	
G2/3	188 (37.6)	9 (32.1)		2 (14.3)		2 (16.7)		3 (60.0)	
Cases of LN metastasis	265 (53.0)	19 (67.9)	0.089	7 (50.0)	0.968	9 (75.0)	0.111	1 (20.0)	0.194
No. of LN metastasis, mean + SD	2.8 + 5.1	4.4 + 6.8	0.223	5.2 + 9.5	0.341	1.7 + 2.0	0.453	0.2 + 0.4	0.260
Lymphatic invasion, present	133 (26.6)	4 (14.2)	0.307	3 (21.4)	0.385	4 (33.3)	0.771	0 (0)	0.161
Vascular invasion, present	17 (3.4)		0.357	0 (0)	1.000	0 (0)	1.000	0 (0)	1.000

IDC, invasive ductal carcinoma-not otherwise specified; ILC, invasive lobular carcinoma; SD, standard deviation; NAC, neoadjuvant chemotherapy; ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor receptor 2; G, grade; CR, complete response; PR, partial response; PD, progressive disease; pCR, pathological complete response; LN, lymph node.

cases was 500 and 62. Table 2 shows the NAC regimens that were administered. Prior to NAC, the average age and tumor size were not significantly different for the different groups. The HG was significantly higher in the IDC group ($p < 0.001$). The immunohistochemical findings and ER and PgR status were not significantly different in the two groups, however, the HER2 status was more frequently positive in the IDC group ($p = 0.006$). After NAC, the SP group was significantly less likely to achieve a clinical response ($p = 0.002$) and had tumors that were larger in size ($p < 0.001$). There were 20 patients who discontinued NAC because of PD. This was 11.3% of the cases in the SP group, which was significantly higher ($p = 0.003$) than that in the IDC group. BCT was performed significantly more often for IDC patients than SP patients (53.0% vs. 25.8%, $p = 0.019$). Axillary lymph node metastasis was present in 53.0% of patients in the IDC group and 61.3% of those in the SP group, which was not significantly different. The average number of metastatic lymph nodes was not significantly different between the groups. With regard to the pathological response, the pCR rate was 22.6% in the IDC group and 8.1% in the SP group, which was not significantly different. The rate of pathological response grade 3 was also not significantly different between the groups. However, 9.7% of SP patients had no pathological response, and this was a significantly higher rate than that in IDC patients ($p = 0.021$). The IDC group had larger tumors ($p = 0.042$), and more severe ($p = 0.018$) lymphatic invasion. The frequency of vascular invasion was not significantly different between the groups. According to a multivariate analysis, the significantly different characteristics in the SP group were a larger tumor size after NAC, more frequent HER2-negative status, more PD and a lower severity of lymphatic invasion (Table 3).

Histological classification and clinicopathological response to NAC

According to the WHO classification, squamous cell carcinoma, spindle cell carcinoma, carcinoma with cartilaginous and/or osseous metaplasia, and matrix-producing carcinoma were included in the category of metaplastic carcinomas (MPC). The total number of MPC was 14 cases. The tumor size of mucinous carcinomas, MPC and apocrine carcinomas was only minimally reduced, and this was significantly different from IDC ($p = 0.001$, $p = 0.003$ and $p < 0.001$). The clinical response of MPC was significantly poorer than that of IDC ($p = 0.003$) and a half of MPC cases

Table 5
The results of the multivariate analysis of the patient and tumor characteristics of patients with metaplastic carcinomas and mucinous carcinoma (WHO).

	Metaplastic		Mucinous	
	OR (95% CI)	p value	OR (95% CI)	p value
Tumor size, Prior NAC			1.416 (0.983–2.041)	0.062
Tumor size, After NAC	1.443 (1.065–1.956)	0.018	1.226 (0.765–1.964)	0.398
ER positive	0.122 (0.012–1.265)	0.079	1.746 (0.350–8.703)	0.496
PgR positive	0.389 (0.042–3.603)	0.406		
Histological grade	5.935 (0.709–49.680)	0.100	0.077 (0.021–0.280)	<0.001
Clinical response, (CR + PR)	0.545 (0.125–2.367)	0.418		
Clinical response, CR	0.117 (0.001–35.290)	0.830	0.071 (0.001–20.076)	0.861
Clinical response, PD	36.409 (3.408–289.011)	0.003		
Pathological response, pCR	0.028 (0.001–27.724)	0.835	0.003 (0.001–17.390)	0.898

OR, odds ratio; CI, confidence interval; NAC, neoadjuvant chemotherapy; ER, estrogen receptor; PgR, progesterone receptor; CR, complete response; PR, partial response; PD, progressive disease; pCR, pathological complete response.

developed PD, which was significantly higher than the rate of IDC ($p < 0.001$). The HG was lower in mucinous carcinomas ($p < 0.001$) and higher in cases of MPC ($p = 0.016$) than in IDC (Table 4). A multivariate analysis indicated that mucinous carcinoma had a lower HG and that MPC had a larger tumor size after NAC and more frequently developed PD than did patients with IDC (Table 5).

Prognosis after treatment and histological features

The patient survival was evaluated using a median follow-up period of 49 months (range, 1–136 months). The 10 year DFS rate was 28% in the SP group and 62% in the IDC group ($p < 0.001$). The OS was significantly worse in the SP group than the IDC group ($p < 0.001$). The incidence of recurrence or death was also significantly higher in the SP group (OR. 2.359; 95% CI, 1.443–3.856; $p < 0.001$ and OR, 4.825; 95% CI, 2.473–9.412; $p < 0.001$, respectively). The independent risk of recurrence or death was analyzed using a Cox multivariate analysis (Table 6). The independent risk factors for recurrence were a younger age, a high HG and the presence of lymphatic invasion. The pathological response grade was a significant factor associated with OS. However, PD was not a significant factor for predicting the DFS or OS.

According to the WHO classification, the DFS and OS of MPC and ILC were significantly worse than those of IDC. However, there were no cases of recurrence or death in the patients with apocrine carcinoma (Fig. 1). The incidence of recurrence or death was significantly higher in the MPC group (OR, 3.076; 95% CI, 1.057–8.951; $p = 0.031$ and OR, 7.053; 95% CI, 2.347–21.197; $p < 0.001$, respectively). The other three types were not significantly different with regard to the incidence of recurrence or death. Because there was only a small number of cases of each histological type, no significant independent risk factor for recurrence or death were identified in the multivariate analysis of each histological type.

Discussion

For breast cancer patients, NAC has been standardized for the purpose of reducing the tumor or for downstaging the tumor. For IDC, standardized NAC regimens have been established, and the effects of treatment have been widely shown.[16,17] However, because of their rareness, the therapeutic effect and outcome after NAC for other types (excluding IDC) were unclear, and standardized regimens for each histological type have not been established. In Japan, standardized NAC was started in 1998, and has been administered for all types of invasive breast carcinoma. We have demonstrated that there are differences in the clinicopathological effects and outcomes after NAC for different types of invasive breast carcinoma, and that these differences are especially pronounced between IDC and other minor types based on the Japanese and the WHO classifications.

Although the SP group had a significantly poorer outcome with regard to tumor reduction and the pathological response, there were actually two sub-types of tumors; those that were effectively reduced by NAC (mucinous carcinoma, ILC and apocrine carcinoma) and those that increased in size despite treatment (squamous carcinoma and spindle cell carcinoma). Under the WHO classification, these increased types were included among the MPC group.

Overall, the SP group had a significantly poorer prognosis than the IDC group. However, according to the WHO classification, the SP group could be sub-classified into better and worse prognostic types, irrespective of the poor response to NAC. ILC and MPC had significantly poorer outcomes than IDC, but mucinous carcinoma and apocrine carcinoma did not have significant differences in their DFS and OS compared to IDC patients. These results suggest that the SP group in the JBCS classification includes different biological and clinical types.

The behavior and a better prognosis of mucinous carcinoma and apocrine carcinoma were reported.[18–21] Because of their better prognosis regardless of the little effect of NAC, the role of NAC for these carcinomas was limited and NAC might not be needed.

MPC was characterized that the biological and clinical malignancies,[22,23] and the subgroups of MPC included carcinoma with cartilaginous and/or osseous metaplasia and matrix-producing carcinoma were previously reported by Wargotz et al.[24–28] Because of its sarcomatous lesion, MPC has only a minimal response to NAC using the conventional regimens[29] and the effeteness of anti-sarcoma regimens including ifosfamide and etoposide was reported.[30] In our study, the clinicopathological characteristics and response to NAC were similar to other reports,[31–34] but the prognosis was poorer and different. From 1990

Table 6
The hazard ratio of the disease free interval and overall survival in patient with special types based on the multivariate Cox regression analysis.

	DFS			OS		
	HR	95% CI	*p* value	HR	95% CI	*p* value
Age	0.898	0.832–0.969	0.005	0.979	0.885–1.082	0.673
Tumor size						
Prior NAC	0.874	0.537–1.424	0.589	1.273	0.884–2.415	0.084
After NAC	1.166	0.696–1.956	0.559	1.604	0.948–2.713	0.078
Stage	0.815	0.154–4.305	0.810	0.914	0.241–5.214	0.897
Hormone receptors						
ER positive	1.416	0.197–10.187	0.730	0.383	0.040–3.687	0.406
PgR positive	3.540	0.449–27.927	0.230	0.547	0.018–17.071	0.731
HER2 positive	0.007	0.001–3.142	0.974	0.071	0.001–4.682	0.991
Histological grade	6.022	1.458–24.864	0.013	3.195	0.312–31.992	0.330
Clinical response						
Responded (CR + PR)	0.480	0.029–7.985	0.609	0.555	0.072–40,281	0.572
CR	0.004	0.001–6.486	0.991	0.013	0.001–9.246	0.995
PD	4.628	0.353–60.629	0.243	4.560	0.221–92.262	0.326
Pathological response, pCR	0.871	0.001–17.512	1.000	0.653	0.032–12.486	0.998
Pathological response grade	0.754	0.314–1.811	0.528	0.339	0.117–0.983	0.046
Cases of LN metastasis	1.084	0.091–12.867	0.949	1.898	0.032–23.623	0.868
No. of LN metastasis	1.111	0.949–1.301	0.188	5.856	0.031–52.465	0.889
Lymphatic invasion, present	6.384	1.329–30.666	0.021	2.243	0.225–22.394	0.491
Vascular invasion, present	12.136	0.001–144.730	0.964	4.467	0.001–35.241	0.994

DFS, disease-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; NAC, neoadjuvant chemotherapy; ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor receptor 2; CR, complete response; PR, partial response; PD, progressive disease; pCR, pathological complete response; LN, lymph node.

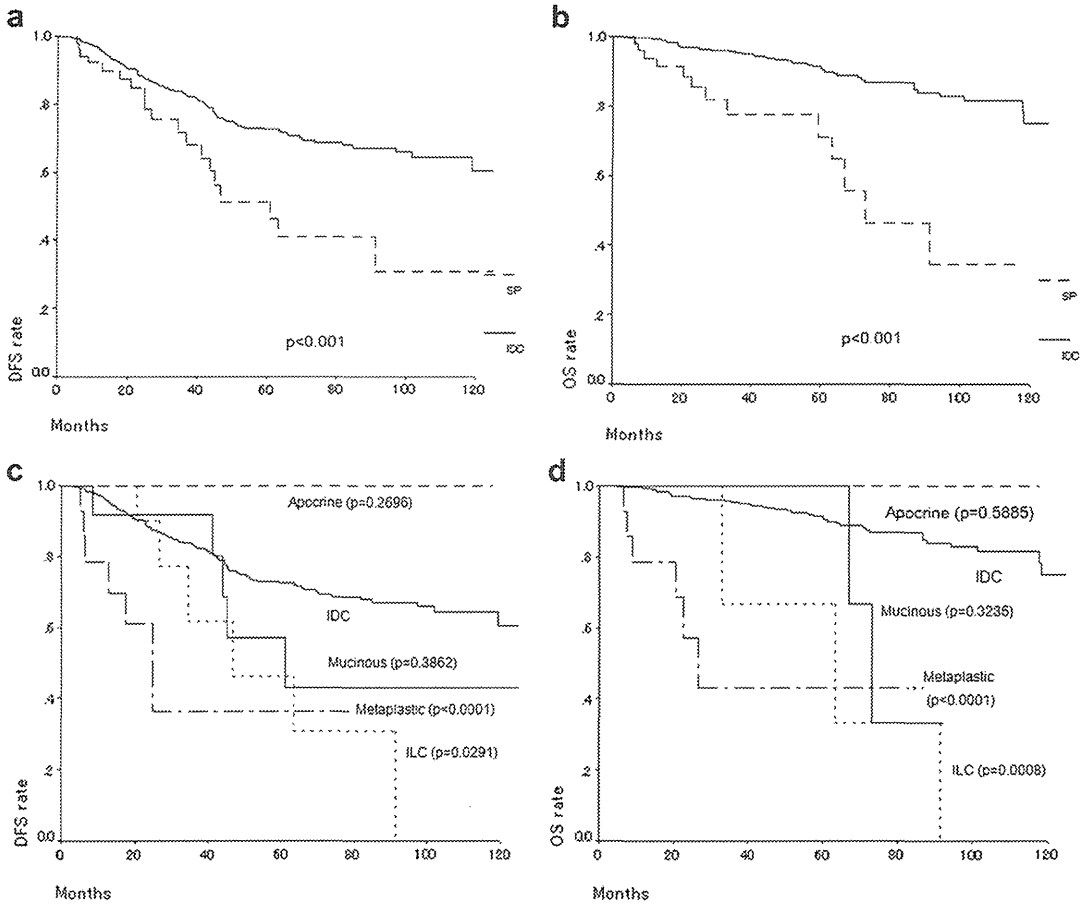

Fig. 1. The disease-free survival (DFS) curves and overall survival (OS) curves. (a) The DFS of IDC and SP patients based on the JBCS classification, (b) The OS of the IDC and SP patients, (c) The DFS for each histological type based on the WHO classification, (d) The OS for each histological type.

to 2009 at our institute, the 10-year survival rate of IDC and ILC patients were 81.8% and 76.5%, which was not significantly different. The reason for the relatively poor prognosis in our study is unclear, but it is possible that the chemosensitivity of ILC may differ in different races as a result of genetic differences.

Currently, breast cancer has been shown to be classifiable into molecular sub-types by gene profiling, and these sub-types related to different prognoses.[35,36] The use of adjuvant or neoadjuvant therapies has been shifting from an emphasis on the histological type to being based on the specific molecular sub-types. With regard to the molecular sub-types, positivity for the ER and/or PgR was not associated with any significant difference between the IDC and SP groups, but there were significantly more HER2-negative cases in the SP group. Several authors have reported that HER2-positive tumors were predicted to have an improved response to chemotherapy and to achieve a much higher pCR rate.[37,38] The HER2-negative status may be one reason why the SP group had a poorer overall response to NAC.

The relationship between chemosensitivity and the molecular sub-types has already clarified that ER-negative tumors have a good response to chemotherapy.[38,39] The molecular sub-types, prognosis and epidemiology of each rare histological type were reviewed by Yerushalmi et al.[40] From the analysis of the histological type in our study, the ER status was found to be positive in cases of mucinous carcinoma and negative in cases of MPC. ER positive status is considered to be the reason for the poor response of mucinous carcinoma. However, MPC had poor response to NAC regardless of the ER status, so the reason for the poorer prognosis is still unclear. MPC is considered to be a basal-like tumor because it is 'triple-negative', and this type has poor chemosensitivity and a poor prognosis.[41] In fact, all of the PD cases in our SP group were MPC. Because of their poor response, NAC is generally omitted for these patients, and surgical resection is performed as the primary therapy for mucinous carcinoma and MPC.

Besides molecular sub-types, other classifications, such as that using the 21-gene expression profile assay and 70-gene assay, have been used for predicting the response to neoadjuvant and adjuvant therapy.[42,43] Although a review concerning the relationship between neoadjuvant endocrine therapy and the 21-gene expression profile assay was reported from Japan,[44] this was a pilot study, and the scoring tools are not yet widespread because of the high price of employing this method. New therapeutic regimens based on the further analysis of the relationship between the immunohistological features or gene expression profiles and therapeutic sensitivity are thus needed.

Some of the limitations associated with this study are the fact that it was a retrospective analysis, and the study population was small due to the rareness of patients with each histological type in the SP group. Trastuzumab therapy was performed in only 52 cases, although there were 109 cases with HER2-positive tumors. The reason for this difference is the date of approval of trastuzumab in Japan. Chemotherapy regimens have been changed during the period of the study, and a uniform evaluation of the effects of therapy cannot be performed. Additionally, treatment for breast cancer has been changed dramatically in the past few years.[45] Because the basis of treatment has been changed from histopathological characteristics of tumor or the presence or absence of lymph node metastasis to intrinsic sub-type of tumor, the role of

chemotherapy has been getting smaller. Therefore the treatment criterion in this review may be different.

In summary, the other minor types of invasive breast carcinoma were different from IDC with regard to the effects of NAC and the prognosis. To determine whether NAC should be administered for the various sub-types of breast cancer, an accurate histological diagnosis and an appreciation of the individual sub-type's sensitivity and responsiveness to NAC are essential. Favorable chemotherapy regimens should be developed for each sub-type. For the types with poor response to NAC, innovative regimens based on their unique clinicopathological features should be investigated.

Conflict of interest

None declared.

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Original article

Sentinel lymph node biopsy using indigo carmine blue dye and the validity of '10% rule' and '4 nodes rule'

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ABSTRACT

This is the study which assessed sentinel lymph node biopsy (SNB) using indigo carmine blue dye and the validity of the '10% rule' and '4 nodes rule'. Patients (302) were performed SNB using the combined radioisotope (RI)/indigo carmine dye method. Excised SLNs were confirmed whether they were stained and numbered in order of RI count and the percentage of radioactivity as compared to the hottest node was calculated. The relationship between histological diagnosis, dyeing and RI count was assessed. All the patients were detected SLN. Positive nodes were identified in 84 (27.8%) patients and were identified up to the third degree of hottest. All the hottest positive nodes were stained by indigo carmine. From the results, removing the three most radioactive SLNs identified all cases of nodal metastasis without complications. These stopping rules were valid and useful under indigo carmine use too.

Introduction

Sentinel lymph node biopsy (SNB) has been established as the standard operative procedure for axillary staging in patients with early breast cancer.[1,2] SNB benefits patients without metastatic nodes by omitting unnecessary axillary lymph node dissection (ALND) with its concomitant morbidities. Since the introduction of SNB, the required number of nodes to excise to confirm metastasis has been discussed. To reduce the SNB false-negative rate, the number of excised nodes may be increased. However, if many nodes are excised at SNB, it practically approaches ALND and results in increased morbidity and decreased quality of life.

According to the study of SNB with the combined RI/dye method, the ideal number of excised nodes and the methods used to decide on this ideal number were analyzed. The two main procedures, '10% rule' and '4 nodes rule', used to determine the number of excised nodes[3] and the intensity of the radioisotope (RI) count[4] are analyzed. These procedures were studied under SNB using patent blue and isosulfan blue. However, instead of patent blue or isosulfan blue, indigo carmine blue dye has been used for SNB with a combination method in Japan. The aim of this study is to determine the ideal number of nodes for excision under RI and indigo carmine use.

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Materials and methods

Patients

This study is an analysis of 302 patients with clinical stage Tis–T3, node negative breast cancer who underwent SNB at the National Cancer Center Hospital, Tokyo, Japan from October 2008 to November 2009. Patients and tumors characteristics were shown at Table 1. All patients underwent SNB with a combination method using RI and indigo carmine blue dye. This procedure of SNB was approved from Ministry of Health, Labour and Welfare of Japan, and all patients provided written informed consent to be examined in this study.

Sentinel node biopsy

Technetium 99 m sulfur colloid (74 mBq) was injected subdermally into the periareolar area and the area around the primary tumor on the day before surgery. Lymphoscintigraphy was performed immediately after injection and after 3 h. At the time of surgery, 5 mL (20 mg) of indigo carmine blue dye (Daiichi-Sankyo, Tokyo, Japan) was injected subdermally into the periareolar area. SNB was performed by searching for the blue lymphatic stream and radioactivity using a gamma detecting probe (Neoprobe; Neoprobe Corp, Dublin, Ohio). All blue-stained and/or radioactive nodes were excised and regarded as sentinel lymph node (SLN). After removal

Table 1
Clinicopathologic characteristics of the patients and tumors ($n = 302$).

	Number	%
Mean age, years (range)	56.4 (27–86)	
Menopausal status		
Premenopausal	109	36.1
Postmenopausal	193	63.9
Tumor laterality		
Right	166	55.0
Left	136	45.0
Tumor location		
Upper inner quadrant	90	29.8
Lower inner quadrant	40	13.2
Upper outer quadrant	126	41.7
Lower outer quadrant	42	13.9
Central	4	1.3
T stage		
Tis	39	12.9
T1	145	48.0
T2	109	36.1
T3	9	3.0
Tumor histology		
DCIS	39	12.9
Invasive ductal carcinoma	243	80.5
Invasive lobular carcinoma	16	5.3
Other	4	1.3

SD, standard deviation; and DCIS, ductal carcinoma in situ.

of the nodes, SLNs were confirmed whether they were stained by dye and were measured the radioactivity *ex vivo*. SLNs were numbered in order of RI count and the ratio of each node to the hottest node was calculated. All nodes with 10% or more of the *ex vivo* count of the hottest node were evaluated intraoperatively by frozen section. If metastases were identified in the SLNs, ALND was performed. In this study, patients with isolated tumor cells were considered to have SLN metastasis for which ALND was performed. ALND was omitted for patients without metastatic nodes.

Pathological examination

For frozen sections, the SLN was sectioned in the center. After the intraoperative frozen section, all nodes were submitted for permanent sectioning. The SLN was sectioned as close to 2–3 mm as possible, and processed with hematoxylin and eosin staining and immunohistochemistry using anti-cytokeratin antibodies (CAM 5.2 and AE1:AE3). Patients with metastases detected by either method were considered to be positive. If the metastatic lesion was between 0.2 and 2.0 mm in size, the node was defined as having micrometastasis. Macrometastasis was defined as a lymph node with metastatic lesions over 2.0 mm. Isolated tumor cells were defined as a lymph node with metastatic lesions less than 0.2 mm.

For comparison of categorical variables, the chi-square test was used. A p-value of 0.05 was considered statistically significant. All data were analyzed using SPSS software (SPSS Inc., Chicago, IL).

Results

In this study, SLN was successfully identified in all patients. More than one SLN was identified in 239/302 (82.5%) patients. The mean number of SLNs excised was 2.6 (range, 1–6). There were 84 patients with positive SLNs (27.8%) and 59 patients with only one positive SLN. The mean number of positive SLNs was 1.3 (range, 1–4). Total number of positive SLNs was 105. The number of positive node detected by RI and dye was 79 (75.2%). The rest was detected by RI or dye only and 24 (22.9%) were not stained and 2 (1.0%) were not detected by RI (Table 2). Table 3 shows the relationship between histological diagnosis and the order of RI count.

Table 2
The procedures of operation and the results of sentinel lymph node biopsy.

	Number	%
Surgery		
Total mastectomy	130	43.0
Lumpectomy	172	57.0
Total number of SLNs excised	782	
Mean number of SLNs excised (range)	2.6 (1–6)	
Number of positive SLNs	84	27.8
One positive SLN only	59	19.5
Total number of positive SLNs	105	
RI and dye	79	75.2
RI only	24	22.9
Dye only	2	1.0
Mean number of positive SLNs (range)	1.3 (1–4)	

SLN, sentinel lymph node; and RI, radioisotope.

Of the 105 total histological positive SLNs, 71 (67.6%) were the hottest node. All metastatic nodes were covered to the fifth degree of RI count. The most radioactive positive node of each patient was diagnosed up to the third hottest node (Fig. 1). Isolated tumor cells were only found in the hottest node. For each patient, the percentage of each node's RI count to the hottest node was calculated. When a RI count of 10% of hottest node is used as the cut-off, the proportion of positive patients captured by SNB was 94.1% and the false-negative rate was 5.9% (Fig. 2). All the hottest positive nodes were stained by indigo carmine. There was no complication associated with SNB.

Discussion

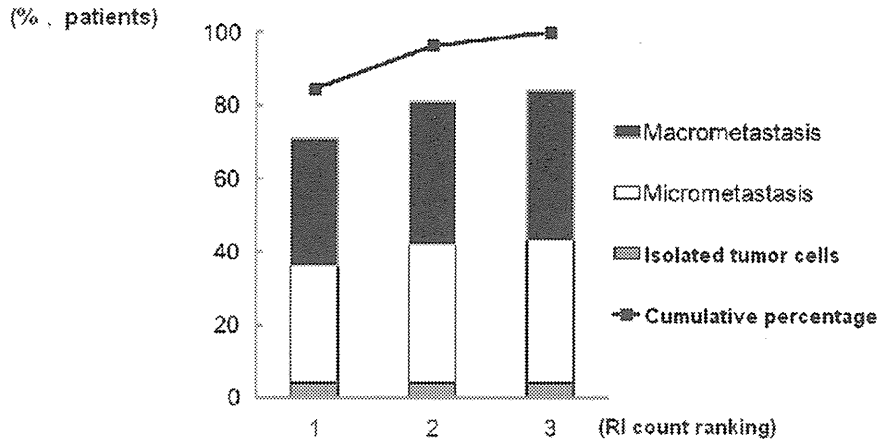
In the surgery for breast cancer patients, the theory of SLN has been established and since its introduction, SNB has allowed patients with negative biopsies to skip ALND and its associated morbidities. Though the procedure of SNB has been standardized by surgical oncologists, the ideal number of nodes to excise remains a question. In these stopping rules, patent blue or isosulfan blue was used. Instead of patent blue and isosulfan blue, indigo carmine has been used and can be used for SNB safety in Japan. This study investigated the ideal number of nodes to excise which satisfy a low false-negative rate under the use of indigo carmine blue dye.

Indigo carmine is the diagnostic dye and has been used for renal function test. Its molecular mass is 466.4 and near patent blue and isosulfan blue. Although Albo et al. and Montgomery et al. reported that anaphylactic reaction for isosulfan blue was appeared in 1.1–1.6% of patients,[8,9] there was no report of serious side effect with indigo carmine.

In this study, all positive nodes were captured up to the fifth rank in radioactivity, and the most radioactive positive node was captured up to the third rank in radioactivity. The optimal number of excised nodes was reported by some authors. McCarter et al. reported that 99.1% of patients were captured positive node up to the fourth site.[3] Almost all other studies reported that the only positive SLN is rarely identified beyond the fourth sampled node.[5] Since metastasis can be found in the 3–5th most radioactive SLN,

Table 3
Histological diagnosis of all sentinel lymph nodes in order of decreasing radioisotope count.

	1st	2nd	3rd	4th	5th
Isolated tumor cells ($n = 4$)	4	0	0	0	0
Micrometastasis ($n = 42$)	32	7	2	1	0
Macrometastasis ($n = 59$)	35	14	5	3	2
Total	71	21	7	4	2



	1	2	3
Isolated tumor cells (n=4)	4	4	4
Micrometastasis (n=39)	32	38	39
Macrometastasis (n=41)	35	39	41
Total	71	81	84
Cumulative percentage (%)	84.5	96.4	100

Fig. 1. In patient with positive node ($n = 84$), the most radioactive positive node was within third rank of radioisotope count.

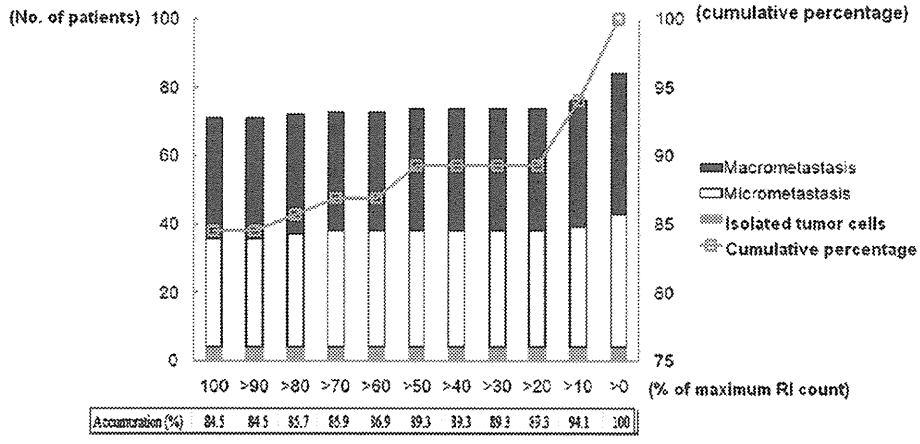


Fig. 2. Histological diagnosis according to percentage of the maximum radioisotope count.

and the TNM classification requires 6 or more lymph nodes to be excised for pN staging, the boundary between SNB and ALND can be defined at the number 6. Another guideline known as the '10% rule' was introduced in 2000 by Martin et al.[4] According to this rule, any node with 10% or more of the *ex vivo* RI count of the hottest node should be removed as a SLN. When the '10% rule' was used in this study, the false-negative rate was 5.9% and slightly high. The reported false-negative rates were 1.7% in Chung et al. [6] and 5.8% in Martin et al.[4] The accepted false-negative rate for SNB was 5%[7] and a record of less than 5% false-negatives at our institute since the introduction of SNB in 2000.

Although 22.9% of positive nodes were not stained but detected by RI only, all the hottest positive nodes were stained by indigo carmine. According to the current procedure of SNB, all the nodes that were stained and/or react to the gamma probe were excised by surgeon. Therefore the number of excised node by RI was apt to larger. And dye method is inferior to RI or combination method in detective rate, generally.[10,11] However, Narui et al. reported that a 4 node sampling method using only patent blue was reliable,[12] and in our study 2 nodes were found only through the dye method not due to accumulate RI. Metastatic disease can injure the lymphatic system and technical errors can occur during SNB. Recognition of the method's limitations and careful intraoperative palpitation are necessary.

In summary, the validity of these stopping rules in SNB under the use of indigo carmine blue dye was analyzed. Though the '10% rule' resulted in slightly high false-negative rate with the RI count method, a positive sentinel lymph node was identified in 100% of cases within the first 3 sentinel nodes when indigo carmine and RI was used. Under the use of indigo carmine, terminating SNB at 3 sampled nodes was the validity procedure to minimize the false-negative and complication rates.

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/

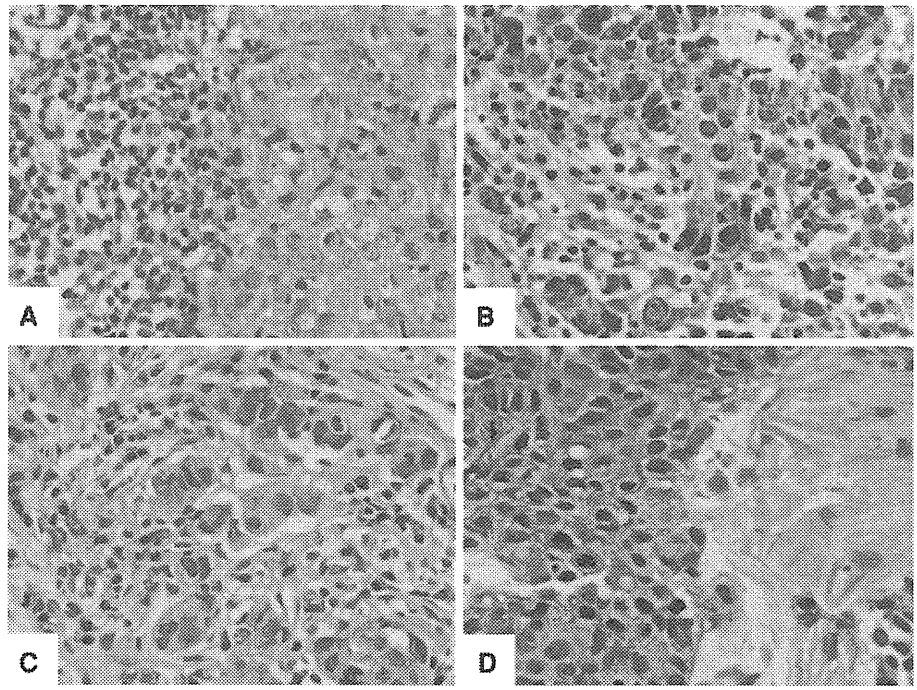

Fig. 1 Histophathological features of tumor-infiltrating lymphocytes (TILs). **a** High TIL score (proportional score 3+ intensity score 2); **b** High TIL score (proportional score 2+ intensity score 2); **c** Low TIL score (proportional score 1+ intensity score 2); **d** Low TIL score (proportional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

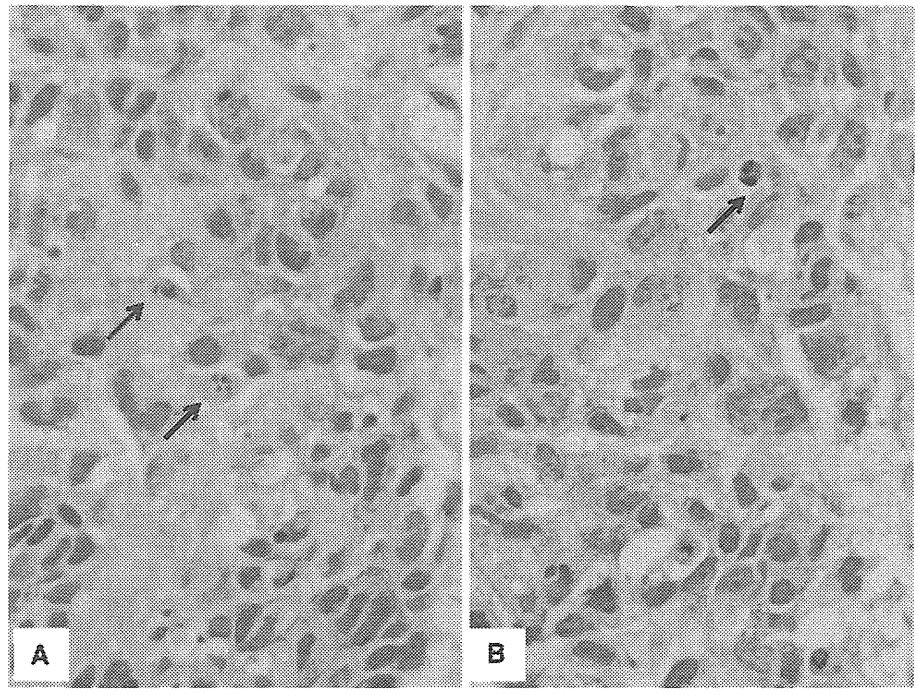

Fig. 2 Histophathological features of breast carcinoma with apoptosis (**a**, **b**) (*arrows*: apoptosis) Original magnification: 400×

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