Original Article

Long-Term Outcomes of S-1 Combined With Low-Dose Docetaxel as Neoadjuvant Chemotherapy (N-1 Study, Phase II Trial) in Patients With Operable Breast Cancer

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Abstract

We analyzed the long-term outcomes and developed the candidate predictors of pathological complete response (pCR) in patients with operable breast cancer receiving S-1+docetaxel. The 5- year disease-free and overall survival (OS) rates were 80.7% and 90.9%, respectively. Patients who achieved pCR had a longer OS rate than non-pCR patients. High tumor-infiltrating lymphocytes, nuclear grade 2 to 3, and some miRNAs could be predictors of pCR predictors.

Background: We previously reported that S-1 and low-dose docetaxel (DOC) (N-1 study, phase II trial) could be a well-tolerated and effective neoadjuvant chemotherapies (NACs) for patients with operable breast cancer. Herein, we analyzed the long-term outcomes and developed clinicopathological and molecular predictors of pathological complete response (pCR). **Patients and Methods:** Eighty-three patients received S-1 (40 mg/m^2 orally on days 1-14) and DOC (40 mg/m^2 intravenously on day 1) every 3 weeks for 4 to 8 cycles. Disease-free survival (DFS) and overall survival (OS) were analyzed for each population with a pCR status. To assess the relationship between pCR and clinicopathological factors such as tumor-infiltrating lymphocytes (TILs, 1+ <10%, 2+ 10%-50%, and 3+ >50%) and nuclear grade (NG), microarray was used to compare the microRNA profiles of the pCR and non-pCR groups using core needle biopsy specimens. **Results:** With a median follow-up duration of 99.0 (range, 9.0-129.0) months, the 5-year DFS and OS rates were 80.7% and 90.9%, respectively. The 5-year OS rate of the pCR group was significantly better than that of the non-pCR group (100% vs. 86.2%, $p = .0176$). Specifically, in triple-negative patients, the difference was significant (100% vs. 60.0%, $p = .0224$). Multivariate analysis revealed that high TILs (≥ 2–3+) and NG 2–3 independently predicted pCR. Microarray data revealed that 3 miRNAs (miR-215-5p, miR-196a-5p, and miR-196b-5p) were significantly upregulated in the pCR group. **Conclusion:** Our NAC regimen achieved favorable long-term outcomes and significantly improved OS in the pCR group. High TILs, NG 2–3, and some miRNAs may be predictors of pCR.

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Introduction

Neoadjuvant chemotherapy (NAC) is a standard treatment for breast cancer. Specifically, pathological complete response (pCR) is a surrogate marker of prognosis.[1-4] The pCR rate of NAC for patients with human epidermal growth factor receptor 2 (HER2)-negative primary breast cancer is 13% to 22%.[1] In addition, there is a 20% to 30% risk of recurrence in patients who fail to obtain pCR

from NAC with anthracycline followed by taxane, which currently remains a standard regimen.[3,4]

Anthracycline followed by a taxane regimen does not yield a satisfactory pCR rate, especially in patients with luminal-type breast cancer.[4] In addition, anthracyclines can cause severe bone marrow suppression and cardiotoxicity. In particular, cardiotoxicity due to anthracyclines may negatively affect the prognosis and treatment continuity of patients, even if the cancer is cured.[5] Therefore, anthracyclines can sometimes be extremely harmful to patients with breast cancer.

We conducted a phase II trial (N-1 study) of S-1 (Taiho Pharmaceutical Co., Tokyo, Japan) combined with low-dose docetaxel (DOC) as a new NAC regimen without anthracyclines.[6] Subsequently, we reported that this N-1 study could achieve a good response rate (80.7%) and pCR rate (34.9% overall, 50.0% in the triple-negative (TN) type, and 19.5% in the luminal type). These results could be comparable to those of anthracycline followed by a taxane regimen.[3,4] Furthermore, 81.9% of patients could take S-1 at >80% of the required dose, and the adverse events were acceptable. Finally, we concluded that our regimen was well tolerated and used as a new NAC regimen in patients with operable breast cancer.[6]

MicroRNAs (miRNAs) are small (approximately 22 nucleotides) noncoding RNAs, that regulate gene expression at the posttranscriptional level.[7] miRNAs can regulate the expression of various downstream gene targets, such as oncogenes and tumor suppressor genes, and play important roles in cancer initiation and progression.[8] Recently, several studies have reported on the roles of miRNAs in predicting the response to NAC in patients with breast cancer.[9-11]

Here, we aimed to report on the long-term outcomes of patients treated in this study and identify the clinicopathological factors and candidate miRNAs to predict the achievement of pCR with this regimen.

Patients and Methods
Patients, Study Design, and Consolidated Standards of Reporting Trials Diagram

A diagram of the Consolidated Standards of Reporting Trials diagram is shown in Figure 1. The details of the N-1 study design, patient characteristics, treatment protocols, and primary efficacy results have been previously reported.[6] In brief, from May 2009 to December 2013, 83 patients with operable breast cancer were enrolled. The distribution of the 4 breast cancer subtypes was as follows: luminal (estrogen receptor [ER]+ and/or progesterone receptor [PgR]+, HER2–), n = 41 (49.4%); luminal HER2 (ER+ and/or PgR+, HER2+), n=13 (15.7%); HER2 (ER– and/or PgR–, HER2+), n=13 (15.7%); and TN (ER– and/or PgR–, HER2–), n = 16 (19.3%). The treatment schedule is shown in Supplementary Figure 1. All patients received low-dose DOC (40 mg/m^2) administered intravenously on day 1 and oral S-1 (40 mg/m^2) on days 1 to 14 every 3 weeks for 4 cycles. According to the Response Evaluation in Solid Tumors criteria, 5 patients who achieved a complete response underwent surgery, and 57 patients who achieved a partial response received 4 additional cycles of S-1+DOC. Among the 21 patients who had stable or progressive disease, 12 received epirubicin and cyclophosphamide for 4 cycles,

and 9 patients received trastuzumab and paclitaxel according to their HER2 status.[6]

This study was not preplanned. However, because of the good outcomes achieved in the previous study,[6] we designed this additional follow-up study. In this study, the primary endpoints were overall survival (OS) and disease free survival (DFS). The secondary endpoints were late adverse events and recurrence patterns. In addition, to investigate the clinicopathological factors and candidate miRNAs associated with pCR, we also performed histological assessment and miRNA microarray analysis. This study was approved by the Institutional Review Board of the Tokushima University Hospital, Tokushima, Japan (no. 4477). All patients provided informed consent.

Histological Assessment of Nuclear Grade, Ki-67 Expression, and Tumor-Infiltrating Lymphocytes

Nuclear grade (NG), Ki-67 expression, and tumor-infiltrating lymphocytes (TILs) were histologically assessed using core needle biopsy (CNB) before NAC. NG was divided into 3 groups in order of increasing atypia: NG 1, NG 2, and NG 3. This grading system is routinely used in Japan because it is associated with the prognosis of Japanese patients with breast cancer.[12] Ki-67 was assessed using immunohistochemistry. Regarding the evaluation of TILs, we adopted a method of evaluating the area ratio (%) of TILs in the tumor stroma standardized by the International Immuno-Oncology Biomarker Working Group, which is organized mainly by TIL researchers.[13] The percentage of stroma with TILs among the total intratumoral stroma was calculated with no information about the patients' clinical course. TILs were evaluated within the borders of the invasive cancer nests, excluding the outside of the tumor border, and around the carcinoma in situ and the normal ducts and lobules. A full assessment of the average TILs in the tumor area was performed; however, we did not focus on hotspots. We subsequently categorized the results into 3 grades: TILs 1+ <10%, TILs 2+ 10% to 50%, and TILs 3+ >50%, as the categorization could most easily reach consensus. The histological findings were confirmed by 2 experienced pathologists (BY and UH).

Follow-Up Examination

In principle, follow-up examinations were conducted for 10 years along a planned schedule. The follow-up included a physical examination, mammography, tumor marker measurement, and ultrasound. Tumor marker levels were routinely checked every 6 months. Mammography or ultrasound were routinely performed every 12 months. Additional computed tomography (CT), ^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography (FDG-PET/CT) or brain magnetic resonance imaging (MRI) was performed if there were unfavorable events.

Prognosis

All time-to-event endpoints were defined as the time in months from the date of breast cancer diagnosis. DFS was calculated from the time of diagnosis to recurrence (locoregional or distant, contralateral breast cancer excluded) or death from any cause, whichever occurred first. OS was calculated from diagnosis to death

Figure 1 Flow diagram of this study. Abbreviations: DOC, docetaxel; cCR, clinical complete response; cPR, clinical partial response; cSD, clinically stable disease; cPD, clinical progressive disease; HT, trastuzumab and taxane; EC, epirubicin and cyclophosphamide; TN, triple-negative.
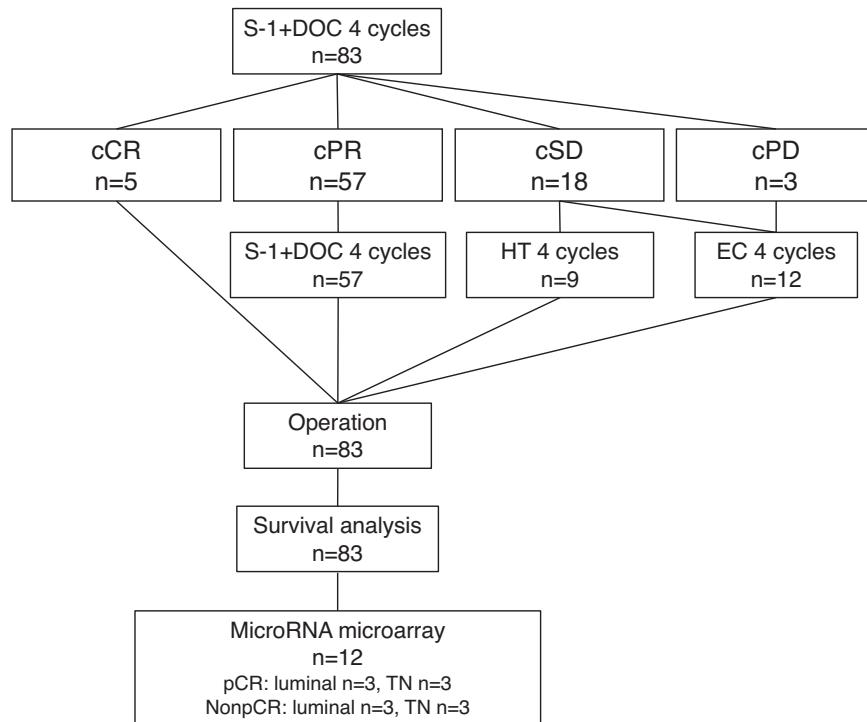
from any cause or the last follow-up. Patients without events were censored on the date of last contact.

MicroRNA Microarray

Twelve breast tumor specimens obtained by CNB before treatment were subjected to miRNA microarray analysis. The details were as follows: luminal (pCR [n = 3] vs. non-pCR [n = 3]) and TN (pCR [n = 3] vs. non-pCR [n = 3]) (Supplementary Table 1). No significant differences were observed between the pCR and non-pCR groups.

Breast tumor sections (10 μm) were prepared from each formalin-fixed paraffin-embedded specimen. The paraffin was removed by xylene treatment, and the tissues were washed twice with ethanol to remove xylene. The tissues were subsequently treated with proteinase K at 37°C overnight. After centrifugation, the supernatant was processed using a silica-based spin column (Toray Industries, Inc., Tokyo, Japan) to obtain purified total RNA. The degree of RNA cross-linking and degradation was analyzed via electrophoresis using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA). The concentration of the RNA solution was determined by measuring the absorbance at 260 nm.

The extracted total RNA was labeled using a 3D-Gene miRNA labeling kit (Toray Industries Inc., Tokyo, Japan). The labeled RNAs were hybridized onto a 3D-Gene Human miRNA Oligo Chip (Toray Industries Inc., Tokyo, Japan). The annotation and oligonu-cleotide sequences of the probes conformed to those in the miRBase miRNA database (http://microrna.sanger.ac.uk/sequences/). After stringent washes, the fluorescent signals were scanned using a 3D-Gene Scanner (Toray Industries, Inc., Tokyo, Japan) and analyzed using the 3D-Gene Extraction software (Toray Industries, Inc., Tokyo, Japan).

The raw data for each spot were normalized by substitution with the mean intensity of the background signal, determined by all blank spots' signal intensities at 95% confidence intervals (CIs). Measurements of spots with signal intensities >2 standard deviations of the background signal intensity were considered valid. The relative expression levels of each miRNA were calculated by comparing the signal intensities of the valid spots throughout the microarray experiments. The data were globally normalized per array, such that the median signal intensity was adjusted to 25.

Statistical Analyses

All the values are expressed as the means and ranges. Cumulative DFS and OS rates were determined using Kaplan–Meier plots and log-rank tests. Fisher's exact test was used to determine significant differences in the relationship between pCR and clinicopathological factors. Logistic regression was used for univariate and multivariate analyses. We performed univariate analyses of pCR for six factors: TILs, HR, T stage, N stage, age, and NG. We also used the stepwise method for multivariate analysis to evaluate the following

2 variables: TILs 2–3+ vs. TILs 1+ and NGs 2–3 vs. NG 1. A p-value <0.05 was considered significant in all instances. JMP version 14 (SAS Institute Inc., Cary, NC) was used for the statistical analyses.

Results

Surgical Procedures, Postoperative Treatments, and Late Adverse Events

Patient characteristics are shown in Table 1. Breast-conserving surgery (BCS) was performed in 66 (80%) patients, and mastectomy was performed in 17 (20%) patients. Sixty-five of the 66 patients (99%) who underwent BCS received postoperative radiation therapy. Four of the 17 patients (24%) who underwent mastectomy received postmastectomy radiation therapy. Fifty-three of 54 patients (98%) in the luminal and luminal HER2 subtypes received adjuvant endocrine therapy. Twenty of the 26 patients (77%) in the luminal HER2 and HER2 subtypes received adjuvant trastuzumab.

There were no late adverse events that reduced quality of life, including cardiac events, during the follow-up period.

Prognosis

After a median follow-up duration of 99.0 (range, 8.8-129.0) months, 17 DFS events, including 10 deaths, were reported. Overall, 15 distant relapses and 2 locoregional relapses occurred as the first events.

The 5-year DFS and OS rates were 80.7% and 90.9%, respectively (Figures 2A, 3A). The 5-year DFS rates were 87.9% and 74.8% in the pCR and non-pCR groups, respectively ($p = .1288$). There was no significant difference; however, the DFS rates tended to be better in the pCR group than in the non-pCR group. In particular, the 5-year DFS rate of luminal patients in the pCR group was greater (100%) than that of patients in the non-pCR group (81.3%, $p = .1965$, Figure 2C). Similar trends were also observed in the TN patients (72.9% vs. 46.9%, $p = .2561$, Figure 2d). In contrast, there were no significant differences in the other subtypes (Supplemental Figure 2A, B). However, the 5-year OS rate of the pCR group was significantly better than that of the non-pCR group (100% and 86.2%, respectively; $p = .0176$; Figure 3B). Specifically for TN patients, the 5-year OS rate of the pCR group was significantly better than that of the non-pCR group (100% vs. 60.0%, $p = .0224$; Figure 3D). However, there were no significant differences in the other subtypes (Supplemental Figure 2C, D), although the OS rates tended to be better in the pCR group than in the non-pCR group (luminal: 100% vs. 90.6%, $p = .3449$; Figure 3C).

TILs and Pathological Complete Response (pCR)

TILs could be evaluated in 66 patients. The patients were divided according to TIL grade was as follows: TILs 1+, n = 52 (78.8%); TILs 2+, n = 11 (16.7%); and TILs 3+, n = 3 (4.5%). The pCR rates were 17.3% (9/52) in the TILs 1+ group, 63.6% (7/11) in the TILs 2+ group, and 100% (3/3) in the TILs 3+ group. A significant relationship was observed between TILs and pCR ($p = .0002$). TILs were divided into 2 groups: The TILs-low group (TILs 1+, n = 52) and TILs-high group (TILs 2–3+, n = 14) according to the scoring system.[14] The TILs-high group had a significantly greater pCR rate than the TILs-low group (71.4% [10/14] vs. 17.3% [9/52],

$p = .0002$). Additionally, among patients (n=51) who were treated with only S-1 and DOC, the TILs-high group had a significantly greater pCR rate (66.7%, 8/12) than the TILs-low group (20.5%, 8/39) ($p = .0047$).

NG and pCR

NG could be evaluated in 62 patients. The distribution of patients according to NG was as follows: NG 1, n = 27 (43.5%); NG 2, n = 21 (33.9%); and NG 3, n = 14 (22.6%). The pCR rates were 11.1% (3/27) in the NG 1 group, 38.1% (8/21) in the NG 2 group, and 64.3% (9/14) in the NG 3 group. A significant relationship was observed between NG and pCR ($p = .0033$). NG was further divided into 2 groups, the NG 1 group (n = 27) and the NG 2 to 3 group (n = 35), according to the scoring system.[15] The NG 2 to 3 group showed a significantly greater pCR rate than the NG 1 group (48.6% [17/35] vs. 11.1% [3/27], $p = .0018$). Additionally, among patients (n = 45) who were treated with only S-1 and DOC, the NG 2 to 3 group showed a significantly greater pCR rate (50.0%, 13/26) than the NG 1 group (11.8%, 2/19) ($p = .0055$).

Determination of the Correlation Between Clinicopathological Factors and pCR Using Logistic Regression Models

Univariate analyses demonstrated that TILs (odds ratio [OR], 11.94; 95% CI, 3.05-46.73; $p = .0002$), NG (OR, 7.56; 95% CI, 1.92-29.77; $p = .0016$), and T factor (OR, 0; $p = .0001$) were significantly associated with pCR. Multivariate analysis of TILs, the ER, and the NG indicated that TILs (OR, 46.76; 95% CI, 4.74-461.62; $p = .0010$) and NG (OR, 14.95; 95% CI, 1.46-153.22; $p = .0227$) were independent predictive factors of pCR (Table 2).

Comparison of the Expression Profiles of microRNAs Between pCR and non-pCR

The detected miRNAs were clustered and are shown as a heat map (Figure 4). We extracted 14 miRNAs (fold change >1.50 or fold change <0.67), including 3 upregulated and 2 downregulated miRNAs in the TN group and 5 upregulated and 4 downregulated miRNAs in the luminal group (Table 3). Among these, 3 miRNAs were significantly regulated. MiR-215-5p was downregulated in the TN group, and miR-196a-5p and 196b-5p were downregulated in the luminal group. In contrast, 203a-3p was downregulated in the TN group, but upregulated in the luminal group, although these differences were not significant.

Discussion

This is the first study to demonstrate the long-term outcomes of NAC with S-1+DOC as a regimen without anthracyclines for operable breast cancer treatment. Our results indicated that the 5-year DFS and OS rates were 80.7% and 90.9%, respectively, which could be comparable or even superior to those of previous randomized controlled trials in which anthracyclines were used as a standard regimen.[3,16] In addition, we also emphasize that our regimen is well-tolerated and has little cardiotoxicity.

In this study, the overall pCR rate was 34.9% (50.0% in the TN group and 19.5% in the luminal group). In patients who achieved pCR, the 5-year DFS rate was 87.9%, which was better compared

Table 1	Patient Characteristics				
Characteristics	**Associated Characteristics**	**Total (n = 83)**	**pCR (n = 29)**	**Non-pCR (n = 54)**	**_p_ Value**
Age	Mean (range)	55 (24–75)	56 (31–75)	55 (24–75)	.7546
cT	2	65	29	36	.0012
	3	8	0	8	
	4	10	0	10	
cN	Positive	65	23	42	.5531
	Negative	18	6	12	
cStage	II	63	29	34	.0001
	III	20	0	20	
ypT	0	13	15	1	.0001
	Is	14	14	0	
	1	32	0	29	
	2	14	0	14	
	3	4	0	4	
	4	6	0	6	
ypN	0	60	29	36	.0004
	1	20	0	15	
	2	2	0	2	
	3	1	0	1	
ER	Positive	54	15	39	.0638
	Negative	29	14	15	
PgR	Positive	43	11	32	.0628
	Negative	40	18	22	
HER2	Positive	26	13	13	.0519
	Negative	57	16	41	
Subtype	Luminal	41	8	33	.0311
	Luminal HER2	13	7	6	
	HER2	13	6	7	
	Triple-negative	16	8	8	
NG	1	27	3	24	.0033
	2	21	8	13	
	3	14	9	5	
	Unknown	21	9	12	
Ki-67 (%)	≤20	11	4	7	.0060
	>20	30	18	12	
	Unknown	42	13	29	
TILs	Low (1+)	52	9	43	.0001
	High (2–3+)	14	10	4	
	Unknown	17	10	7	
Surgical procedures	BCS	66	25	41	.2685
	Mastectomy	17	4	13	
LN dissection	SLNB	10	4	6	.7339
	ALND	73	25	48	
Adjuvant therapy	RT (+)	69	25	44	.7612
	RT (−)	14	4	10	
	ET (+)	53	12	41	.0086
	ET (−)	30	17	13	
	Tmab (+)	20	11	9	.0030
	Tmab (−)	63	18	45	
Follow-up period (months)	Mean (range)	99 (9-129)	96 (9-129)	100 (10-129)	.9050

Abbreviations: pCR = pathological complete response; NG = nuclear grade; TILs = tumor-infiltrating lymphocytes; BCS = breast-conserving surgery; SLNB = sentinel lymph node biopsy; ALND = axillary lymph node dissection; RT = radiation therapy; ET = endocrine therapy; Tmab = trastuzumab.

Figure 2 Kaplan–Meier survival curves of disease-free survival (DFS). (A) All patients (n=83), (B) pathological complete response (pCR, n = 29) vs. non-pCR (n = 54) among all patients, (C) pCR (n = 8) vs. non-pCR (n = 33) in the luminal subtype (n = 41), and (D) pCR (n = 8) vs. non-pCR (n = 8) in the triple-negative subtype (n = 16).
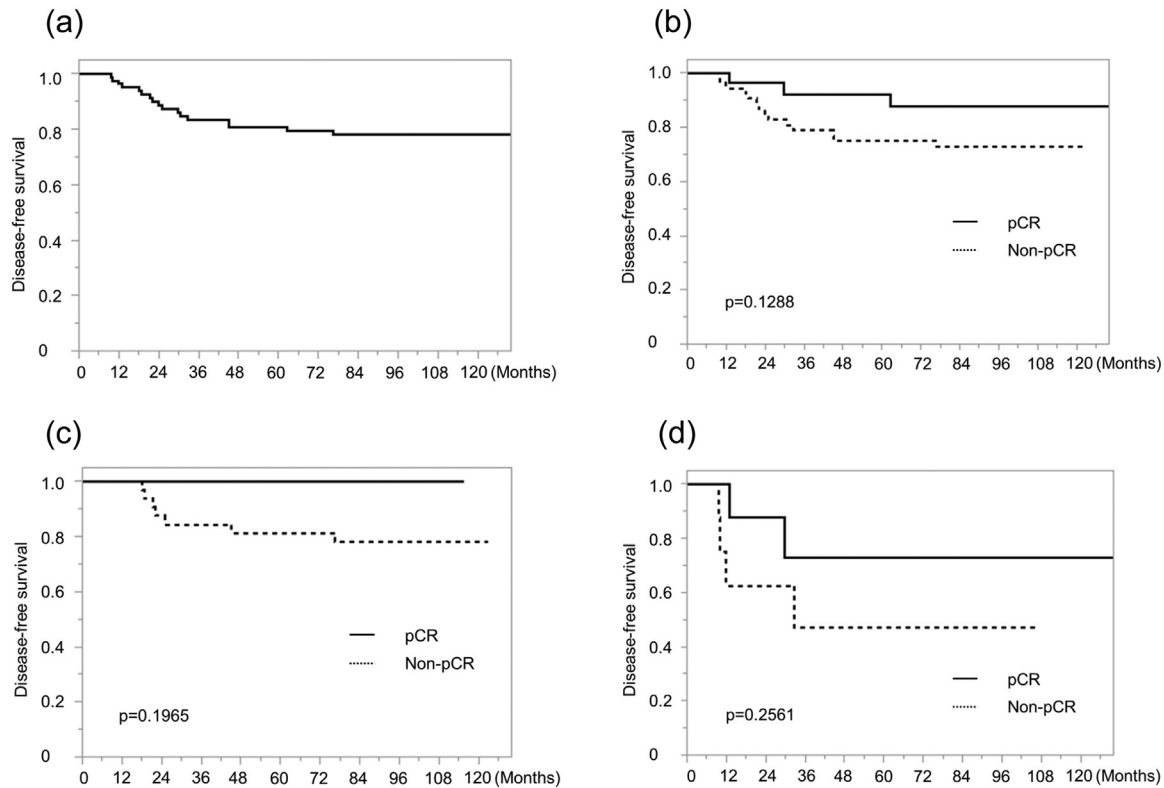
Table 2 Univariate and Multivariate Analyses of the Correlation Between pCR and Clinicopathological Factors

Variables	Univariate		Multivariate	
	OR (95% CI)	*p* Value	OR (95% CI)	*p* Value
TILs high vs. low	11.94 (3.05-46.73)	.0004	46.76 (4.73-461.62)	.0010
N+ vs. N–	1.10 (0.36-3.30)	.8717		
ER– vs. ER+	2.42 (0.95-6.22)	.0648		
NG 2–3 vs. NG 1	7.56 (1.92-29.77)	.0038	14.95 (1.46-153.22)	.0227
S-1 vs. EC or HT	1.47 (0.50-4.33)	.4802		

Abbreviations: pCR = pathological complete response; OR = odds ratio; CI = confidence interval; TILs = tumor-infiltrating lymphocytes; ER = estrogen receptor; NG = nuclear grade; EC = epirubicin and cyclophosphamide; HT = trastuzumab and taxane.

with that of the non-pCR group, although without significance. In this study, anthracyclines were administered to patients who had no or a weak response after 4 courses of S-1+DOC. This was especially evident in the non-pCR group; this may explain why there was no significant difference in the 5-year DFS between the pCR and non-pCR groups. In this study, there were 49 luminal-type patients (59.0%) were enrolled. Unexpectedly, in the pCR group of luminal patients, the 5-year DFS rate was 100%, which was relatively higher and more favorable compared with that of the non-pCR group, although the difference was not statistically significant. S-1, which was a key drug in this study, could also play an important role in NAC for luminal-type breast cancer, as confirmed in the recent POTENT tria.[17] In HER2-positive breast cancer patients, a certain degree of pCR can be achieved with chemotherapy without trastuzumab. However, administering trastuzumab tends to result in a higher pCR rate and improved prognosis. Among the patients included in this study, more than half were not administered trastuzumab, which likely contributed to the lack of differences in prognosis.[2]

The original goal of NAC was to improve the OS. Our results showed that, in patients who achieved pCR, the 5-year OS rate was 100%, which was significantly better than that of the non-pCR

Figure 3 Kaplan–Meier survival curves of the overall survival (OS). (A) All patients (n = 83), (B) pathological complete response (pCR, n = 29) vs. non-pCR (n = 54) among all patients, (C) pCR (n = 8) vs. non-pCR (n = 33) in the luminal subtype (n = 41), and (D) pCR (n = 8) vs. non-pCR (n = 8) in the triple-negative subtype (n = 16).
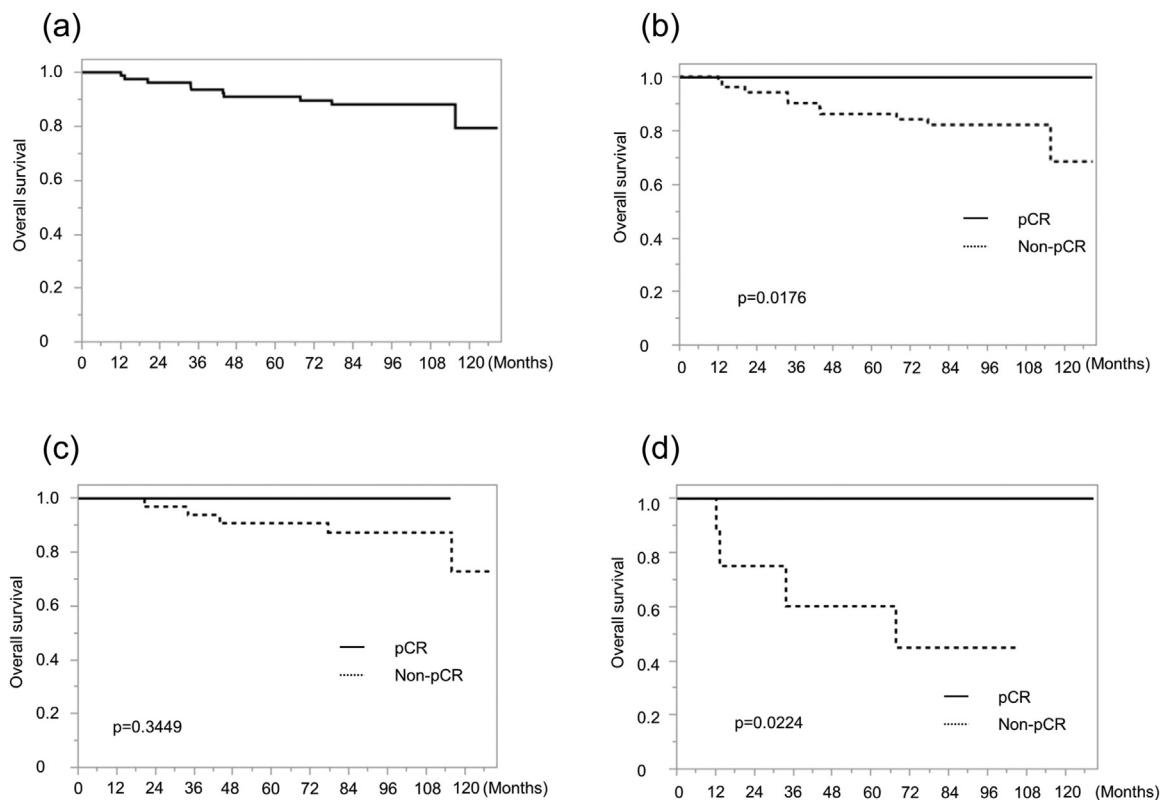

Table 3 Differential microRNA Expressions Between non-pCR and pCR in 6 Patients

MicroRNAs	p Value	Fold Change (>1.5)	Regulation (vs. non-pCR)
Luminal			
has-miR-196a-5p	.022	4.65	Up
has-miR-451a	.382	2.66	Up
has-miR-196b-5p	.036	2.66	Up
has-miR-449a	.468	1.69	Up
has-miR-9-5p	.271	1.63	Up
has-miR-95-3p	.187	1.82	Down
has-miR-203a-3p	.209	1.75	Down
has-miR-338-3p	.155	1.63	Down
has-miR-6720-3p	.054	1.55	Down
Triple-negative			
has-miR-224-5p	.125	2.15	Up
has-miR-203a-3p	.086	2.02	Up
has-miR-215-5p	.004	1.61	Up
has-miR-193a-3p	.158	1.65	Down
has-miR-1297	.311	1.52	Down

Abbreviations: pCR = pathological complete response; miR = microRNA.

Figure 4 A heat map comparing microRNA (miRNA) expression in the pathological complete response (pCR) and non-pCR groups in each luminal and triple-negative (TN) type. We measured 3 samples in each group; miRNAs with blank values were excluded as noise. The extracted genes were clustered as shown.
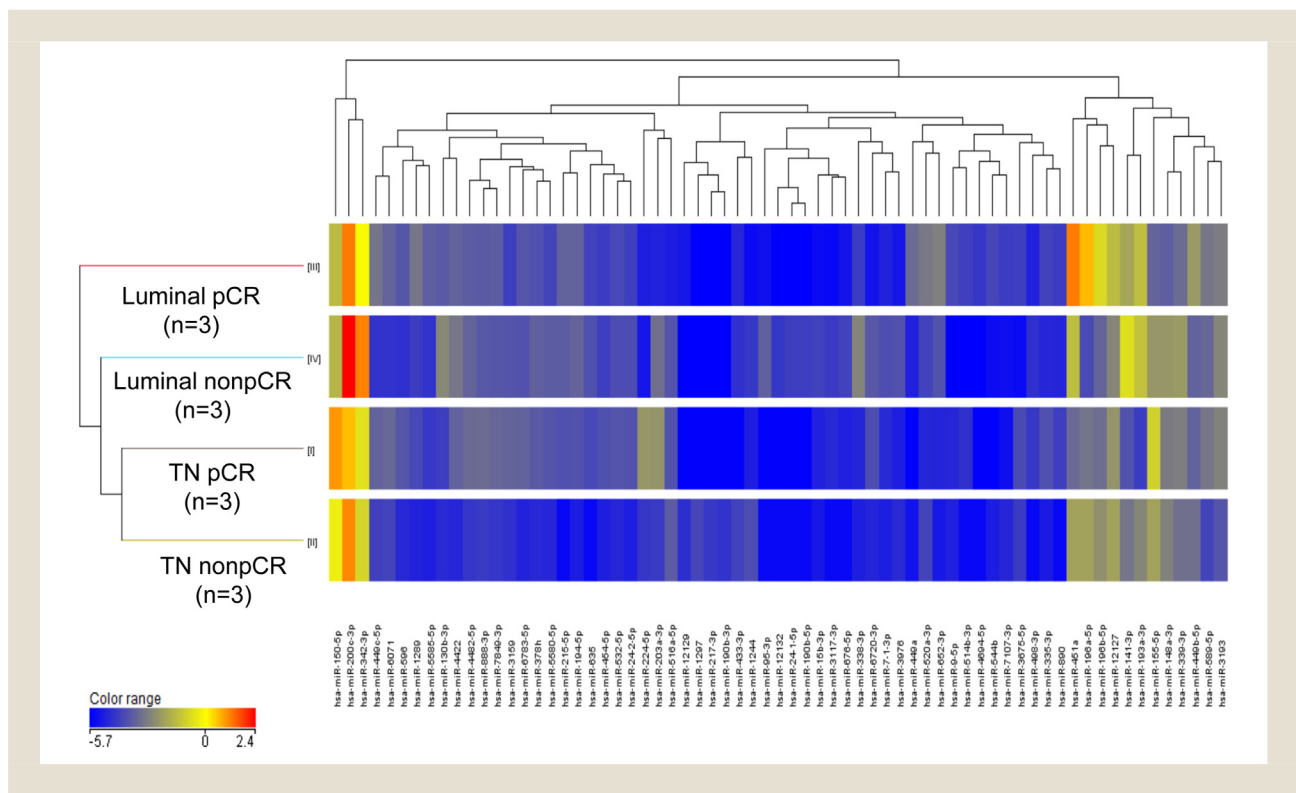
group. Particularly in the pCR group of TN patients, the 5-year OS was significantly better than that in the non-pCR group. pCR was associated with improved OS in a meta-analysis of previous clinical trials of NAC for operable TN breast cancer.[18] Our result was thought to be favorable and consistent with the data from these trials.[18] However, some studies have demonstrated that favorable OS is not achieved even if pCR is achieved. In the NSABP-B27 trial, the pCR rate increased from 13% to 26% after administering anthracycline followed by taxane in the NAC setting. However, there were no significant differences in OS and DFS between the pCR and non-pCR groups.[19] This was because 70% of the patients enrolled had clinically negative lymph node metastases. However, approximately 80% of the patients enrolled in this study had clinically positive lymph node metastases. The OS may have improved because this study was conducted in more advanced patients. Therefore, in terms of improving OS, it might be necessary to fully consider the patient's background, such as nodal status and subtype, in addition to increasing the breast conservation rate.

In this study, multivariate analysis of clinicopathological factors revealed that TILs and NG could be candidate predictors of pCR among clinicopathological factors. TILs are the basic components of the immune system. Several authors have already reported that an increased number of TILs is associated with an excellent prognosis and response to chemotherapy in patients with breast cancer.[20-22] According to systematic review and meta-analysis, higher TIL levels in pretreatment biopsy samples indicated higher pCR rates after

NAC, especially in HER2-positive and TN patients.[23] This finding is consistent with our results. The pCR rate of TN patients was higher (50.0%), which led to improved OS compared with that of patients in the non-pCR group. This is the first study to demonstrate that TILs could be a predictor of pCR in patients who received S-1+DOC in the NAC setting. In this study, to assess TIL scores, CNB specimens before NAC were used according to the evaluation method of the International Immuno-Oncology Biomarkers Working Group.[24] This working group has not yet determined the cutoff value for TILs. However, Adams reported that prognosis improved with each 10% increase in TILs and that the significant cutoff value for TILs could be >10%.[14] In this study, we used this cutoff value and showed that high TILs could be a useful predictor of pCR. Additionally, we demonstrated that NG could be a predictor of pCR with this regimen. Several authors have previously reported that higher NG is associated with chemosensitivity.[25-27] In this study, we compared the pCR rate between NG 1 and NG 2–3 and showed that NG 2–3 could be a predictor of pCR. Therefore, our NAC regimen may be potentially applicable in patients with high-grade tumors evaluated as having high TILs and NG 2–3 using CNB specimens.

In the present study, we focused on miRNAs as potential molecular predictors of pCR. To compare the miRNA expression patterns between the pCR and non-pCR groups in both luminal and TN patients, we comprehensively evaluated the microarrays using CNB specimens. Among the 14 miRNAs extracted (fold change >1.50 or

fold change <0.67), 3 new miRNAs (miR-215-5p, miR-196a-5p, and miR-196b-5p) exhibited significant changes. However, it has not been reported whether these 3 miRNAs can be used as candidate predictors of pCR in NAC patients. In TN patients, miR-215-5p expression was upregulated in the pCR group. MiR-215-5p is a member of the miR-192/215 precursor family that serves as an anti-oncogene in several types of cancer. Upregulation of miR-215-5p is associated with a favorable prognosis in patients with breast cancer and may suppress the aggressiveness of breast cancer cells in vitro by targeting SRY-Box 9.[28] A recent study demonstrated that miR-215-5p could be a predictor of chemoresistance in colorectal cancer,[29] but without reports in TN breast cancer. In contrast, in the luminal type, miR-196a-5p and miR-196b-5p, which are member of the miR-196 precursor family, were upregulated in the pCR group. In patients with breast cancer, miR-196a-5p is overexpressed in the TN type compared with the luminal type.[30] Wang reported that upregulation of miR-196a-5p was associated with chemoresistance in patients with breast cancer using a microarray.[31] However, they did not show the subtypes of patients analyzed, and anthracyclines and cyclophosphamide were administered, unlike in our study. In this study, the upregulation of miR-196a-5p was associated with pCR, which is in contrast to previous data, indicating that regulatory status might change depending on the subtype and regimen administered. Zhu indicated that miR-196b-5p inhibited cell growth and metastasis in breast cancer cells in vitro by targeting the collagen type I alpha 1 chain and could be a promising and effective therapeutic target for breast cancer.[32] miR-196b-5p is associated with chemoresistance in vitro in colorectal cancer cells.[33] However, the expression of miR-203a-3p (fold change >1.50), which is a member of the miR-203 family, was is upregulated in the TN group but downregulated in the luminal group. MiR-203a-3p can enhance the development and oncogenesis of breast cancer by targeting the insulin-like growth factor receptor,[34] but its association with chemosensitivity and chemoresistance has not been reported. Interestingly, it was hypothesized that miR-203a-3p could be a predictor of pCR in both the luminal and TN types, although opposite expression patterns were observed.

This study has several limitations. First, this was a phase II, single-arm, single-institution study, and might be different from modern common treatment for patients with operable breast cancer. However, our regimen achieved favorable long-term outcomes, especially in the pCR group, while ensuring high tolerability. In addition, there have been no clinical trials for all subtypes that have prospectively evaluated long-term prognosis using regimens without anthracyclines as NAC. Therefore, we believe that our regimen can be an option, specifically for patients with high-grade tumors for whom anthracyclines are contraindicated. Second, there were some missing data for TILs (20.5%) and NG (25.3%). Third, the extracted miRNAs were not validated in all the patients. In this study, serum and urine samples were obtained from all patients. Therefore, to develop more convenient predictive miRNAs, further validation studies using serum, urine, and the remaining CNB samples are needed.

Conclusion

The NAC regimen (S-1+DOC) in this study achieved favorable long-term outcomes in patients with operable breast cancer and significantly improved OS, especially in the pCR group. High TILs, NG 2 to 3, and several miRNAs may be predictors of pCR. However, further randomized clinical trials and validation studies are needed.

Clinical Practice Points

- We conducted a phase II trial (N-1 study) of S-1 combined with low-dose DOC as an NAC for patients with operable breast cancer and analyzed its long-term outcomes. At a median follow-up duration of 99.0 (range, 9.0-129.0) months, the 5-year DFS and OS rates were 80.7% and 90.9%, respectively. The 5-year DFS rate of the pCR group was better than that of the non-pCR group (87.9% vs. 74.8%, $p = .1288$). For luminal patients, the 5-year DFS rate of the pCR group was 100%. The 5-year OS rate in the pCR group was significantly better than that of the non-pCR group (100% vs. 86.2%, $p = .0176$). Specifically, in the TN patients, the difference was significant (100% vs. 60.0%, $p = .0224$). Our NAC regimen achieved favorable long-term outcomes and significantly improved OS in the pCR group.
- We also developed clinicopathological and molecular predictors of pCR using CNB specimens. Multivariate analysis revealed that TILs ≥2–3 and NG ≥2 were independent predictive factors of pCR. Microarray data showed that miR-215-5p (fold change=1.61) was significantly upregulated in the TN pCR group, whereas miR-196a-5p (fold change = 4.65) and miR-196b-5p (fold change = 2.66) were significantly upregulated in the luminal pCR group. TILs ≥2–3, NG ≥2, and these 3 miRNAs could be predictors of pCR, although further investigation is needed.

Disclosure

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

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Supplementary Material

Supplementary Table 1	Clinicopathological Characteristics of Patients and Breast Tumors		
Subtype	**pCR**	**Non-pCR**	**_p_ Value**
Luminal (n = 6)	n = 3	n = 3	
Age (years), range	50.3 (36-74)	49 (35-61)	0.9293
Tumor size (mm), range	19.6 (15-23)	35.6 (22-54)	0.1788
Nuclear grade, range	2 (1-3)	1.3 (1-2)	0.3739
Ki-67 (labeling index, %), range	36.6 (10-50)	43.3 (40-50)	0.653
Lymph node metastasis	3	3	1
Postmenopausal	1	1	1
Recurrence	0	0	1
Total courses, range	8 (8-8)	6 (4-8)	0.1583
Triple negative (n = 6)	n = 3	n = 3	
Age (years), range	59.6 (58-63)	63.3 (54-72)	0.5392
Tumor size (mm), range	22 (15-30)	28 (25-32)	0.2581
Nuclear grade, range	2.3 (2-3)	2.3 (1-3)	1
Ki-67 (labeling index, %), range	45 (29-68)	48.6 (26-90)	0.8851
Lymph node metastasis	3	3	1
Postmenopausal	3	3	1
Recurrence	1	3	0.1161
Total courses, range	5.3 (4–8)	6.3 (3-8)	0.6638

Abbreviation: pCR = pathological complete response.

Supplementary Figure 1	Treatment schedule. Abbreviations: DOC, docetaxel; cCR, clinical complete response; cPR, clinical partial response; cSD, clinically stable disease; cPD, clinical progressive disease; HT, trastuzumab and taxane; EC, epirubicin and cyclophosphamide.
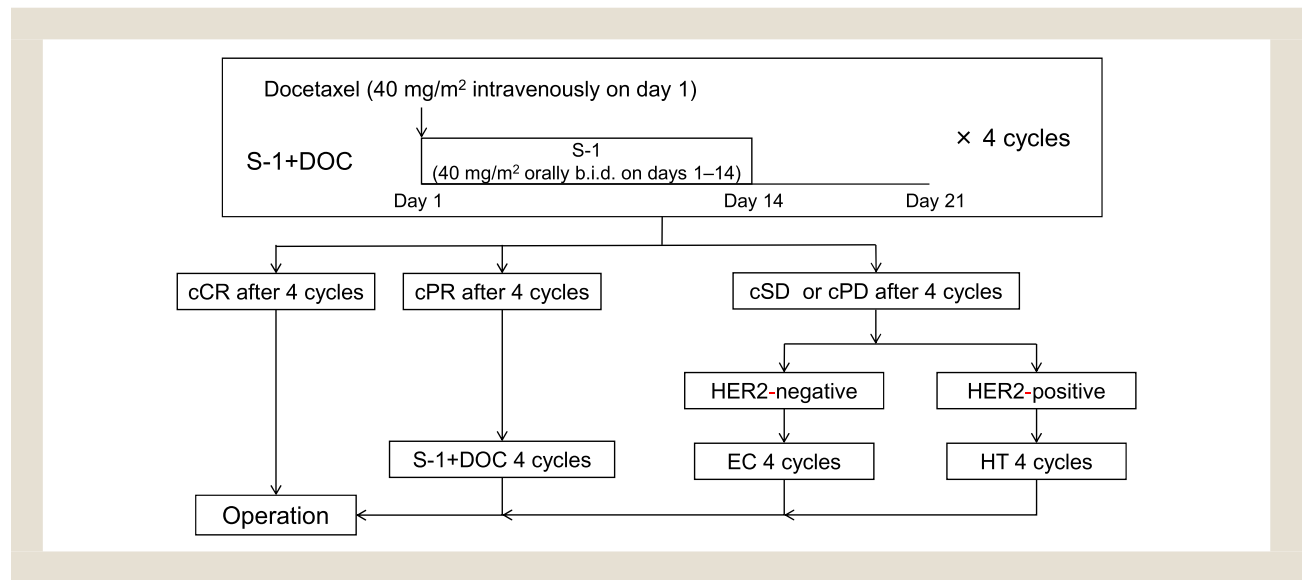
Supplementary Figure 2	Kaplan–Meier survival curves of disease-free survival (DFS) and overall survival (OS) for patients with (a, c) pCR (n = 7) vs. non-pCR (n = 6) in the luminal HER2 subtype (n = 13), and (b, d) pCR (n = 6) vs. non-pCR (n = 7) in the HER2 subtype (n=13).