

Predictive factors for the effectiveness of neoadjuvant chemotherapy and prognosis in triple-negative breast cancer patients

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Abstract

Purpose Triple-negative breast cancers (TNBCs) do not derive benefit from molecular-targeted treatments such as endocrine therapy or anti-HER2 therapy because they lack those molecular targets. On the other hand, TNBCs have been shown to respond to neoadjuvant chemotherapy (NAC). In this study, we analyzed TNBC patients who were treated with NAC at Osaka National Hospital over a recent 5-year period to clarify the predictive factors for NAC and prognostic factors.

Patients and methods Thirty-three TNBC patients underwent sequential NAC with anthracycline (FEC100: 5FU 500 mg/m², epirubicin 100 mg/m², and cyclophosphamide 500 mg/m²/q3w, 4 courses) and taxanes (paclitaxel 80 mg/m²/qw, 12 courses or docetaxel 75 mg/m²/q3w, 4 courses)

from May 2003 to July 2008. Pre-therapeutical and surgical specimens were studied for expressions of ER, PgR, HER-2, EGFR, cytokeratin 5/6, Ki-67, p53 and androgen receptor by immunohistochemistry (IHC). We analyzed clinicopathological factors and molecular markers in regard to the response to NAC and prognosis.

Results Pathological complete response (pCR) was achieved in 12 TNBC patients (36%). The pCR rate in the basal-like phenotype was significantly lower than in the non-basal-like phenotype (23 vs. 64%, respectively: $P = 0.02$). High pre-operative expressions of Ki-67 ($\geq 50\%$) and HER-2 (2+) were considered as predictive factors for a better response from NAC. Pre-operative Ki-67 expression showed a significant correlation with disease-free survival (DFS) and a lower expression of Ki-67 ($< 50\%$) after NAC was favorable for DFS among non-pCR patients.

Conclusions A non-basal-like phenotype and higher expressions of Ki-67 and HER-2 (2+) were favorable factors for NAC. However, a higher expression of Ki-67 on the surgical specimen after NAC was also a poor prognostic factor.

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Keywords Triple-negative breast cancer · Neoadjuvant chemotherapy · Pathological complete response · Ki-67 · Basal-like phenotype

Abbreviations

TNBC	Triple negative breast cancer
NAC	Neoadjuvant chemotherapy
pCR	Pathological complete response
ER	Estrogen receptor
PgR	Progesterone receptor
AR	Androgen receptor
EGFR	Epidermal growth factor receptor
CK	Cytokeratin

Introduction

Triple-negative breast cancers (TNBCs) are characterized by the lack of expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2). These cancers occur in ~20–25% of all breast cancers and are associated with an unfavorable prognosis. They derive no benefit from molecularly targeted treatments such as endocrine therapy or trastuzumab [1]. Therefore, identifying appropriate treatments for TNBC is an important issue.

Recent precise gene expression analysis revealed that TNBC is a heterogeneous group of tumors. One of the subgroups is a basal-like subtype, which is characterized by similar gene expression as the basal/myoepithelial cells of the normal breast [1–5]. Basal-like breast cancer has also been identified with immunohistochemical (IHC) staining of basal markers, such as cytokeratins (CKs) and epithelial growth factor receptor (EGFR). TNBCs without these basal markers are classified as non-basal-like subtypes, which are rare breast cancers, and classifications based on gene expression have not been clarified yet. Non-basal-like tumors are also reported to have a better prognosis than basal-like phenotypes [6, 7]. Because of the lack of targeted therapies and their aggressive clinical behaviors, TNBCs are relevant groups to be investigated for their characteristics. Though TNBCs are considered to have poor prognosis generally, TNBCs have been shown to be chemosensitive.

Neoadjuvant chemotherapy (NAC) in primary breast cancers has been shown to produce an outcome equivalent to that of adjuvant chemotherapy [8, 9]. Patients who show a pathological complete response (pCR) in the primary tumors after NAC have a better prognosis [10]. The pathological responses are important prognostic parameters and can be used as surrogate parameters for clinical outcome, so we analyzed the effects of clinicopathological factors as well as immunohistochemical factors on pathological responses after NAC. However, the paradox that TNBC and HER-2 positive subtypes showed higher chemosensitivity but worse survival due to higher relapse after chemotherapy is also known well [10, 11].

Several biological markers have been proposed as prognostic characteristics in breast cancers. ER, PR and HER-2 are such biological markers as well as being therapeutic markers and Ki-67, p53 and androgen receptor (AR) are shown to be associated with prognosis [12–16]. AR is known to be present in the majority of primary and metastatic invasive breast tumors and is often co-expressed with ER and PR in these tumors. Though little is known about the role of AR in hormonal response, AR expression has been shown to be associated with a better outcome for untreated breast cancer patients [14]. Ki-67 is a nuclear antigen expressed in the G1, S, and G2 phases but not in the

G0 or resting phase of the cell cycle. Ki-67 has been established as a proliferation marker in breast cancers and high proliferation activity has been found to have predictive value for the response to NAC [17]. Also p53 expression status has been used as a predictive factor for response to systemic therapy, because tumor cells with non-functional p53 do not respond to systemic therapy due to a failure in apoptosis [13, 15].

Because chemotherapy is the only treatment other than surgery for TNBC, the definition of clinical markers in regard to chemotherapeutic response and prognosis is very important. However, there are still few studies focusing on TNBC. In this study, we analyzed clinicopathological factors, phenotypes, and molecular markers of TNBC in regard to the response to NAC and prognosis.

Patients and methods

Patients and neoadjuvant chemotherapy

One hundred and 63 breast cancer patients underwent NAC with a sequential regimen containing anthracycline (FEC100: 5FU 500 mg/m², epirubicin 100 mg/m², cyclophosphamide 500 mg/m²/q3w, 4 courses) and taxanes (paclitaxel 80 mg/m²/qw, 12 courses or docetaxel 75 mg/m²/q3w, 4 courses) at Osaka National Hospital (Osaka, Japan) from May 2003 to July 2008. The criteria for entry were invasive breast cancer patients from 20 to 70 years old with any T and N0-2 disease, who were diagnosed histologically, were absent from distant metastasis and with normal organ functions. Thirty-three patients (20%) among 163 breast cancer patients were identified as TNBCs. The clinical evaluation of the response to NAC was determined by clinical findings, CT and MRI examinations according to RECIST. All patients were included in clinical trials approved by an institutional review board and asked for written informed consent.

Immunohistochemistry

Pre-therapeutical specimens were obtained by the 14G-needle biopsy in all cases and pathological examinations using standard hematoxylin and eosin staining were carried out. Immunohistochemical evaluation for ER, PgR, HER-2, EGFR, CK5/6, Ki-67, p53 and AR in tissue sections were detected using antibodies (ER:Cat.No. 760-2596I, PgR: 760-2816, HER-2:760-2901, EGFR:790-2988, CK5/6:960-4253, Ki-67:760-2910, p53:760-2912, Ventana Japan, Yokohama, Japan, AR:M3562, Dako Japan, Tokyo, Japan). Visualization of the bound antibodies was performed using a DAKO EnvisionTM + System (Dako Japan Inc., Tokyo, Japan) according to the manufacturer's instructions. Positive

cell rates (%) of ER and PgR were determined as a ratio of positive cells to total cancer cells and a value of 10% or higher were rated as positive [18, 19]. HER-2 expression was defined as (0) to (3+) based on positive cell rates and the intensity of IHC staining. Tumors showing weak over-expression (2+) of HER-2 were also tested by the fluorescence in situ hybridization (FISH) method to clarify the gene amplification of the *HER-2* gene. The *HER-2* gene is visualized as green fluorescent grains and a control of centromere 17 is visualized as orange fluorescent grains (Path Vysion, Abbott, IL, USA). Thus, HER-2 positives were either strong positives (3+) from IHC or positive for gene amplification from FISH analysis.

TNBCs are negative for ER, PgR and HER-2 as described earlier. Among TNBCs with 1–9% of ER and/or PgR expression were defined as hormone receptor (HR) weak and analyzed separately. TNBCs with HER-2 (2+) and that were FISH negative were also analyzed separately.

Proliferative activity was determined by IHC for the Ki-67 antibody. Ki-67 values were expressed as the percentage of positive cell counts among at least 100 tumor cells in each case. Patients with positive staining of Ki-67 at 50% or more were defined as high Ki-67 patients. AR and p53 were defined as positive if tumor cells showed positive staining regardless of rate. Basal-like subtype was defined as CK5/6 positive and/or EGFR positive in 5% or more cells.

Surgical treatment

All patients underwent surgical treatment after NAC. Breast conservative therapy or a mastectomy with or without axillary dissection was performed according to the decision of the surgeons' conference. Surgical specimens were histologically analyzed again, and the pathological response for NAC was evaluated. When no residual invasive tumor cells were found, tumors were identified as pathological complete response (pCR). Surgical specimens from non-pCR patients were analyzed for expressions of Ki-67, p53 and AR as described earlier.

Statistics

A univariate analysis of the pCR rate was carried out by the χ^2 test, and a multivariate analysis was done by multiple logistic regression analysis. The patients' survival was calculated from the first date of treatment until the date of death or the end of follow-up. A univariate analysis of disease-free survival (DFS) was done using the Kaplan–Meier method with a log-rank test, and a multivariate disease survival analysis was carried out under the Cox proportional hazards model. All data were analyzed with JMP for Windows (SAS Institute, Tokyo, Japan).

Results

Relationship between pCR and clinicopathological factors

Thirty-three patients were identified as TNBCs, and the patients' data are shown in Table 1. The age of the patients ranged from 30 to 68 years old (median 50.0) and 21 patients had clinically positive nodes. Clinical response after NAC was rated as clinical complete response for 14 patients (42%), a clinical partial response for 14 patients (42%), a clinical stable disease for 3 patients (9%), and as a clinical progress disease for 2 patients (6%). Also pCR was achieved in only 12 patients (36%).

The correlations between clinicopathological factors such as tumor size, lymph nodal metastasis, age, histological grade, and pCR rate were analyzed (Table 2). However,

Table 1 Patients' characteristics

Variables	No (%)
Total	33
Age: years-old	30–68 (50 ± 11.1)
Histology	
Papillo-tubular	4 (12)
Solid tubular	14 (42)
Schirrous	11 (33)
Special type	4 (13)
<i>T</i>	
1	1 (3)
2	24 (72)
3	6 (18)
4	2 (6)
<i>N</i>	
0	12 (36)
1	17 (52)
2	4 (12)
Histological grade	
1	1 (3)
2	4 (12)
3	27 (81)
Unknown	1 (3)
HER-2	
0	18 (55)
1+	11 (33)
2+	4 (12)
HR (hormone receptor)	
Negative	26 (79)
Weak	7 (21)

T and *N* were defined by the criteria of UICC-breast

HR weak is a tumor with low levels of ER and/or PgR determined by IHC (1–9% weakly positive cells)

Table 2 pCR ratio based on clinicopathologic and immunohistochemical factors

Variables	Number (%)	pCR (%)	<i>P</i> value	Odd
Age (years old)				
<50	18 (55)	6 (33)	0.69	
50≤	15 (45)	6 (40)		
Size (cm)				
<5	25 (76)	11 (44)	0.09	5.5
5≤	8 (24)	1 (13)		
N				
Positive	21 (64)	8 (38)	0.78	
Negative	12 (36)	4 (33)		
Histological grade				
1–2	5 (15)	3 (60)	0.26	
3	27 (84)	9 (33)		
HR				
Negative	26 (79)	10 (38)	0.95	
Weak	7 (21)	2 (28)		
HER-2				
0, 1+	29 (88)	9 (31)	0.08	6.67
2+	4 (12)	3 (75)		
p53				
Positive	21 (64)	8 (38)	0.78	
Negative	12 (36)	4 (33)		
Ki-67				
50≤ (high)	20 (61)	10 (50)	*0.04	5.5
<50 (low)	13 (39)	2 (15)		
AR				
Positive	6 (18)	3 (50)	0.45	
Negative	27 (82)	9 (33)		
Basal-like [#]				
Positive	22 (67)	5 (23)	*0.02	5.9
Negative	11 (33)	7 (64)		
CK5/6				
Positive	14 (42)	2 (14)	*0.02	
Negative	19 (58)	10 (53)		
EGFR				
Positive	18 (55)	4 (22)	0.06	
Negative	15 (45)	8 (53)		

* Statistically significant

[#] Basal-like subtype is defined as CK5/4 positive and/or EGFR positive. Thus, CK5/6 was not used for multivariate analysis

these clinicopathological factors did not show any correlation with the pCR rate.

Relationship between pCR, and molecular markers

Next, the correlation between molecular markers and the pCR rate was also analyzed. HER-2 (2+) tended to show a

higher pCR rate than HER-2 negative (0 or 1+; 75 and 31%, respectively). In this study, basal markers of CK5/6 and EGFR were evaluated with 22 of 33 patients (67%) diagnosed with basal-like phenotype, and eleven patients (33%) diagnosed with the non-basal-like phenotype. The pCR rate for the basal-like phenotype was significantly lower than in the non-basal-like phenotype (23 and 64%, respectively; $P = 0.02$; Table 2). Ki-67 was also considered as a predictive factor for NAC response, because the pCR rate reaches 50% among high Ki-67 ($\geq 50\%$) patients, while it was 15% in low Ki-67 patients ($P = 0.04$). The expressions of HR, p53 and AR were not correlated with pCR in this study. Multivariate analysis showed that only high Ki-67 was a significant factor for the prediction of pCR (Table 3). The classification of basal-like or non-basal-like phenotypes was negative for multivariate analysis, probably because high Ki-67 and non-basal-like were strongly correlated with each other; high Ki-67 accounted for 33% in the basal-like and 75% in the non-basal-like phenotype.

Relationship between pCR and disease-free survival

All patients underwent surgical resection after NAC and non-pCR patients were histologically evaluated. The average observation period after surgery was 2 years and eight patients (24%) showed distant metastasis during the observation period. Seven out of 8 patients had been defined as non-pCR and only one patient obtained pCR after NAC. Non-pCR patients showed a worse DFS compared with pCR patients, but it was not statistically significant (Fig. 1a). Basal-like phenotype and other clinicopathological factors such as age, tumor size and lymph nodal involvement failed to show a correlation with DFS (Table 4). Ki-67 before NAC showed a significant correlation with DFS and high Ki-67 patients showed a poor prognosis (Fig. 1b).

Disease-free survival among non-pCR patients

Among non-pCR patients, only 7 patients (29%) showed a recurrence. We analyzed clinicopathological and IHC factors for better prognosis among non-pCR patients. The immunohistological changes of tumors after NAC were

Table 3 Multivariate analysis of pCR and immunopathological factors

Variables	Odds	<i>P</i> value
Non-basal-like	3.9	0.13
HER2 (2+)	10.2	0.12
High Ki-67	8.4	0.03*

* Statistically significant

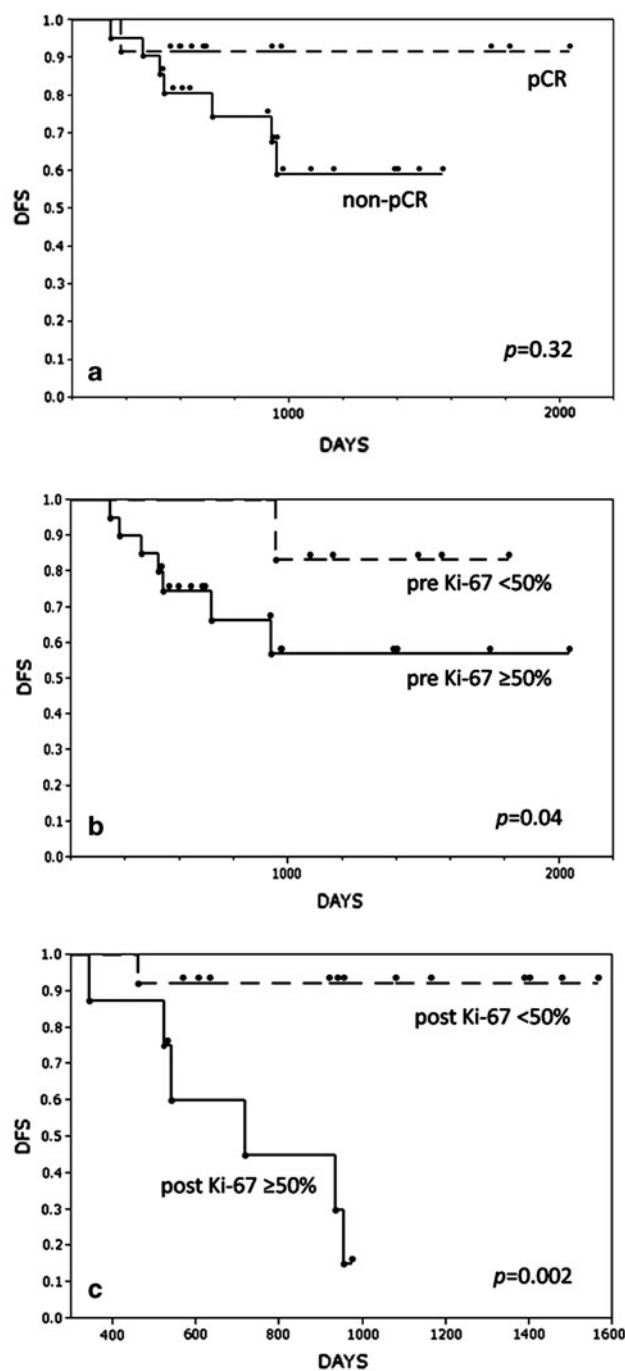


Fig. 1 Disease-free survival (DFS). **a** DFS of pCR and non-pCR patients after NAC. Non-pCR patients showed worse disease-free survival compared with pCR patients, but it was not statistically significant ($P = 0.32$). **b** DFS based on Ki-67 expression of pre-chemotherapy. High Ki-67 ($\geq 50\%$) patients showed significantly worse disease-free survival than low Ki-67 ($< 50\%$) patients ($P = 0.04$). **c** DFS based on Ki-67 expression of post-NAC among non-pCR patients. Non-pCR patients who had high Ki-67 expression after NAC showed a poor prognosis ($P = 0.002$)

evaluated. Among non-pCR patients, 10 patients showed high Ki-67 before chemotherapy and 7 patients still showed high Ki-67 after NAC (Table 5). Among these patients, 6

Table 4 Multivariate analysis of disease-free survival and patients' characteristics

Variables	Hazard ratio	<i>P</i> value
≥ 50 years-old	0.39	0.2
≥ 5 cm	2.2	0.3
N positive	4.2	0.11
HR positive	3.2	0.1
HER-2 (2+)	3.2	0.56
Non-basal	1.4	0.6
High Ki-67	5.95	0.04*
p53 positive	0.48	0.3
AR positive	0.000	0.054
Non-pCR	3.7	0.16
High Ki-67 post-NAC [#]	13.2	0.0029*

[#] Data among non-pCR patients

* Statistically significant

Table 5 The correlation between Ki-67 expression, pCR and the change of Ki-67 expression among non-pCR patients

TNBC (<i>n</i> = 33)	Non-pCR		pCR
	Post-NAC Ki-67		
	High	Low	
Pre-NAC Ki-67			
High	7	3	10
Low	1	10	2

showed a recurrence and Ki-67 values after NAC were significantly correlated with DFS (Fig. 1c). The expressions of p53 and AR after NAC were not correlated with DFS (data not shown).

Discussion

TNBC is defined by the lack of ER, PgR and HER-2 expression. Because targeted therapies are not useful, chemotherapy is the only systemic treatment option for TNBC [1–5]. Thus, a comprehensive examination of the clinical phenotypes of TNBCs which respond to chemotherapy is important. TNBCs are a heterogeneous group and generally divided into two subtypes; basal-like phenotype and non-basal-like phenotype [6]. The basal-like phenotype is characterized as having a high expression of keratins, laminin, and EGFR.

Many data indicated that the pCR rate is higher in TNBC compared with other phenotypes [10]. A pathological evaluation after NAC is very important because pCR after NAC indicates better survival [8, 9]. Our data showed the pCR rate in TNBCs was 36%, which is consistent with previous

reports which stated 22–45% [10, 20]. This study hypothesized that non-basal-like phenotype, HER-2 (2+), and high Ki-67 could be predictive factors for pCR achievement, but multivariate analysis revealed that only Ki-67 was a significant factor for the prediction of pCR. This is probably because the non-basal-like phenotype showed a significantly higher Ki-67 expression compared with the basal-like phenotype. This study is consistent with previous studies which showed that Ki-67 indicates proliferation and high level of proliferation activity are associated with chemosensitivity [14]. Additionally, there are many reports that showed that the basal-like phenotype has a positive correlation with pCR [20]. Rouzier et al. reported that basal-like subtypes were more sensitive to NAC than luminal and normal-like cancers, but normal-like subtypes classified based on gene expression profiles are quite different from non-basal-like phenotypes based on IHC, because normal-like subtypes involved 60% of ER positive samples. Because classification based on gene expression is difficult for clinical use, our data based on IHC classification are quite useful. There are some reports that non-basal-like tumors showed better prognosis than basal-like phenotypes [6, 7]. Though the pCR rate was significantly higher in non-basal-like tumors, there was no difference in DFS between the two groups in this study.

Our study failed to show the significant benefit of pCR on DFS. That is probably because of the small number of the patients included or the short duration after surgical treatment in this study. Most cases which showed a recurrence in such a short period were non-pCR patients, and the only recurrent case in the pCR group was a patient with an intraductal residual after NAC and who showed brain metastasis within a year. In this study, Ki-67 was the only significant factor which was proved to affect DFS. Pre-NAC high Ki-67 was a poor prognostic factor in spite of the positive correlation with pCR. The post-NAC status of Ki-67 was also correlated with recurrence. High Ki-67 expression post-NAC showed a very poor prognosis and low Ki-67 post-NAC showed better survival even in the non-pCR group. The contradiction of high Ki-67 tumors, which showed a high chemosensitivity and high pCR rate but poor prognosis, may indicate the diversity of these tumors. As shown in Table 4, most high Ki-67 patients who could not achieve pCR kept a high expression of Ki-67 after NAC. Tumors which maintained high Ki-67 expression may indicate that the cellular activity is not suppressed by NAC. All of these facts showed that high Ki-67 tumors should be divided into two groups: tumors which show a high sensitivity to current chemo-drugs and a good prognosis and the tumors which continue to have high cellular activity after NAC and show a poor prognosis. Further study is needed to find other treatments for the latter.

Though many reports defined 20–30% of Ki-67 labeling index as a threshold [21], 50% was used for categorization in this study because most TNBCs are positive for Ki-67 and a 50% threshold at 50% was shown to be useful to predict both chemosensitivity and prognosis in TNBC patients.

The prognosis of HER-2 positive breast cancer has been proved by the usage of trastuzumab. The criteria of HER-2 positive are defined as a strong positive IHC or gene amplification in FISH [22]. HER-2 (2+) breast cancers without gene amplification are generally included in TNBC but HER-2 (2+) breast cancers showed higher chemosensitivity in this study and HER-2 (3+) breast cancers have been reported to be chemosensitive. The criteria of HER-2 positivity might be a moot point if TNBCs with HER-2 (2+) show a different cancer biology from TNBCs with negative HER-2.

Less than 10% of hormone receptor positivity had been considered as uncertain endocrine responsiveness or potential resistance [18, 19]. Though tumors with less than 10% hormone receptor positivity were included in TNBCs, we classified those with 0% staining both ER and PgR as HR negative and those with 1–9% as HR weak in this study. But the expressions of HR were not correlated with pCR. Moreover, tumors with any ER positive staining of at least 1% are recommended to be treated with endocrine therapy in latest reports [21, 23]. The categories of highly endocrine responsive and incompletely endocrine responsive are not relevant to the decision for endocrine therapy, but those categories are still important for the decision of chemotherapy.

In this study, we found that the pCR rate for the non-basal-like phenotype was significantly higher than that in the basal-like phenotype, though that difference was negative for multivariate analysis. This is because the positivity of Ki-67 was higher in the non-basal-like phenotype tumors. These data based on classification by IHC are very interesting and informative in a clinical setting because there are some discrepancy between criteria by gene expression profiling and those by IHC. Some previous papers were confused about classification by gene expression and by IHC. Non-basal-like subtype is a term correlated with IHC classification and difficult to adapt to criteria of gene expression. There are few reports focused on the non-basal-like phenotype. Our data may insinuate that non-basal-like subtypes are well adaptive to current chemotherapy and basal-like subtypes need another therapeutic agent. Because our data was based on a small number of patients, further examinations based on IHC classification are needed.

Our study indicated that TNBCs which were found to be non-pCR with high Ki-67 expression after NAC had a poor prognosis. How to treat these TNBCs will be a most important subject for future study. Only chemotherapy is a

proven treatment for TNBCs, but chemotherapy based on anthracyclins and taxanes has not been shown to be enough. There are several studies which showed the efficacy of new chemotherapeutic agents such as carboplatin, bevacizumab and poly (ADP-ribose) polymerase-1 (PARP-1) inhibitor in TNBCs [24–26]. Studies of NAC with these agents are expected to improve the treatment of TNBCs.

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