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ORIGINAL ARTICLE

Predictive value of MGMT, hMLH1, hMSH2 and BRCA1 protein expression for pathological complete response to neoadjuvant chemotherapy in basal-like breast cancer patients

Katsuya Nakai · Hiroyuki Mitomi · Yimit Alkam · Atsushi Arakawa · Takashi Yao · Emi Tokuda · Mitsue Saito · Fujio Kasumi

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Abstract
Purpose To evaluate the importance of biological markers to predict pathologic complete response (pCR) to neoadjuvant chemotherapy (NACT) in patients with locally advanced basal-like breast cancers (BLBCs).
Patients and methods Thirty-two BLBC patients receiving NACT with an anthracycline-based regimen plus taxane were included in this study. The immunoreactivities of MGMT, MLH1, MSH2 and BRCA1 before and after NACT were evaluated.
Results A pCR was obtained in 10 of 32 cases (31%). Cancer-related ($P = 0.013$) and disease-free ($P = 0.023$) survival rates were significantly higher in the pCR group than in the non-pCR group. In biopsy samples before NACT, attenuated expression of MGMT, MLH1, MSH2 and BRCA1 was observed in 12/32 (38%), 0/32 (0%), 5/32 (16%) and 28/32 (88%) cases, respectively. On evaluation of pCR, patients' characteristics (patients' age, menopausal status, or clinical and pathological stages) and immunohistochemical patterns, attenuated expression of MGMT was only found to be significantly predictive of a pCR ($P = 0.018$). Paired biopsy sample before NACT and a surgical tumor material after NACT were available for 19 cases of non-pCR. In these cases, decrease in expression during NACT were more frequently observed for MGMT as compared to MLH1, MSH2 or BRCA1 ($P = 0.021$).
Conclusions MGMT status is a predictive factor for pCR with neoadjuvant anthracycline-based plus taxane combination chemotherapy, which may be helpful in the selection of appropriate NACT for Japanese patients with BLBC.

Keywords Breast cancer · Neoadjuvant chemotherapy · Basal-like subtype · MGMT · Immunohistochemistry · Mismatch repair

K. Nakai · Y. Alkam · E. Tokuda · M. Saito · F. Kasumi
Department of Breast Oncology, Juntendo University School of Medicine, Bunkyo-ku, Tokyo, Japan

H. Mitomi (✉) · Y. Alkam · A. Arakawa · T. Yao
Department of Human Pathology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
e-mail: hmitomi@juntendo-ac.jp

Introduction

In recent studies of breast cancer patients with neoadjuvant chemotherapy (NACT), widely used for patients with locally advanced disease over the last few decades, the pathologic complete response (pCR) proved to be an important independent prognostic indicator for prolonged disease-free and overall survival [1, 2]. Basal-like breast cancer (BLBC) is characterized by triple negative of estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2), and positive for basal cytokeratin, epidermal growth factor receptor (EGFR) or c-Kit [3–5]. The prognosis is generally unfavorable [4, 5]. Although adjuvant hormone therapy has been shown to be effective for ER-positive breast cancers [6] and adjuvant trastuzumab therapy also improves survival with HER2 positive breast cancers [7], no targeted therapy is available for BLBC and chemotherapy is the only option other than surgery. Recent studies have shown that BLBCs are sensitive to NACT [8, 9], but still have an ominous outcome, particularly in patients with poor responses [8].

The mismatch repair genes human mut L homolog (MLH)1 and human mut S homolog (MSH)2 are components of the DNA mismatch repair pathway, whose activation may trigger DNA damage signaling, a process which

induces cell cycle arrest and can lead to cell death [10]. O6-Methylguanine-DNA methyltransferase (MGMT) rapidly reverses alkylation (including methylation) at the O6 position of guanine by transferring the alkyl-group to the active site of the enzyme, constituted by a cysteine [11]. An inactivated MGMT gene allows accumulation of O6-alkylguanine that is the most cytotoxic lesion of alkylating agents, which subsequent to incorrect pairing with thymidine triggers mismatch repair, thereby inducing DNA damage and eventually cell death [12]. In accordance with this mechanism, inactivation of MGMT renders MLH1 and/or MSH2-deficient cells more sensitive to alkylating agents. In sporadic breast cancer, MLH1 and MSH2, but not MGMT are targets of epigenetic silencing and subsequently reduced expression at the protein level in the mismatch repair pathway [13–17].

Breast cancer susceptibility protein (BRCA)1 and BRCA2 are essential for repair of double strand breaks and stalled replication forks by homologous recombination. Accumulated evidence suggests that dysfunction of BRCA1 is a crucial mechanism underlying BLBC tumorigenesis [18, 19], and cells deficient for BRCA1 have been shown to be exceedingly sensitive to poly (ADP-ribose) polymerase (PARP) inhibitors [20, 21]. In addition, PARP inhibitors may also sensitize to alkylating agents such as temozolomide, as suggested in recent trials [22].

The purpose of this study was to evaluate the immunoreactivities of MGMT, MLH1, MSH2 and BRCA1 before neoadjuvant anthracycline-based plus taxane combination chemotherapy and to clarify the potential roles of these proteins in predicting pCR to NACT in Japanese patients with BLBC.

Materials and methods

Patient and materials

Locally advanced breast cancer was defined as breast cancer histologically and/or cytologically documented as stage IIA (T2 $\geq$ 3 cm), IIB, IIIA, IIIB or IIIC using the UICC/TNM classification [23]. A cohort of 412 patients thus selected, receiving preoperative NACT and surgically treated at Juntendo University Hospital (Tokyo, Japan) between 2005 and 2010, were first screened for expression of ER, PgR and HER2 in tru-cut biopsy samples before NACT. In all, 40 cases (9.7%) were negative for three markers (triple negative). Second, triple-negative cases were examined for expression of basal markers (cytokeratin 5/6/14/17 cocktail used), EGFR and c-Kit. Thirty-two cases (80%) of 40 triple-negative cases were positive for at least one of these markers and were therefore considered to be BLBCs [3]. The characteristics of these patients are listed in Table 1.

Table 1 Pretreatment patient and tumor characteristics

Characteristics	No.	%
Total patients	32	
Patient age[a], years		
≤50	13	40.6
>50	19	59.4
Menopausal status		
Premenopausal	12	37.5
Postmenopausal	20	62.5
Initial tumor size		
T1	1	3.1
T2	23	71.9
T3	4	12.5
T4	4	12.5
Initial lymph node status		
N0	13	40.6
N1	14	43.8
N2	3	9.4
N3	2	6.2
Initial clinical stage		
IIA	12	37.5
IIB	13	40.6
IIIA	4	12.5
IIIB	1	3.1
IIIC	2	6.3

[a] Median/mean ± SD (range), years 55.0/52.7 ± 10.8 (33–69)

Neoadjuvant chemotherapy, surgery and follow-up

Patients received NACT with anthracycline-based chemotherapy in 4 cycles of triweekly 80 mg/m^2 of epirubicin and 600 mg/m^2 of cyclophosphamide (EC), or 4–6 cycles of triweekly 500 mg/m^2 of fluorouracil, 75 or 100 mg/m^2 of epirubicin and 500 mg/m^2 of cyclophosphamide (FEC). EC or FEC was followed by taxane chemotherapy in 12 cycles of weekly 80 mg/m^2 of paclitaxel, or 4 cycles of triweekly 75 mg/m^2 of docetaxel. Clinical evaluation of the response to NACT was evaluated prior to surgery after the last cycle of chemotherapy according to the product of primary tumor diameters and the axillary clinical status and was determined by clinical findings, ultrasound, computed tomography and magnetic resonance imaging examinations according to RECIST (Response Evaluation Criteria In Solid Tumors) [24]. The patients were classified into four groups: complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD). After neoadjuvant chemotherapy, patients underwent appropriate surgery according to the size of their residual tumor.

Surgically resected materials as well as tru-cut biopsy samples taken at the time of diagnosis were routinely fixed in 15% formalin and embedded in paraffin wax, according

Table 2 List of primary antibodies used for immunohistochemistry

Antigen	Clone	Source	Dilution	Retrieval	Staining type
ER	SP1	Roche diagnostics	Prediluted	Heat	Nuclear
PgR	1E2	Roche diagnostics	Prediluted	Heat	Nuclear
Her2/neu	4B5	Roche diagnostics	Prediluted	Heat	Membrane
CK5/6	D5/16 B4	DakoCytomation	1:50	Heat	Cytoplasmic and membrane
CK14	LL002	Leica microsystems	1:30	Heat	Cytoplasmic and membrane
CK17	E3	DakoCytomation	1:40	Heat	Cytoplasmic and membrane
EGFR	EGFR 113	Leica microsystems	1:15	Heat	Cytoplasmic and membrane
c-Kit (C-19)	polyclonal (sc-168)	Santa Cruz biotechnology	1:100	Heat	Cytoplasmic and membrane
MGMT	MT 3.1	Thermo scientific	1:40	Heat	Nuclear
MLH1	G168-15	Diagnostic BioSystem	1:50	Heat	Nuclear
MSH2	polyclonal	Calbiochem	1:30	Heat	Nuclear
BRCA1	MS110	Oncogene research products	1:150	Heat	Nuclear

to routine procedures, and sections were cut and stained with hematoxylin and eosin (H&E). All slides stained with H&E were independently examined by two of the authors (H.M. and Y.A.) without knowledge of the demographic or treatment response information. Pathological responses to NACT were evaluated according to previously described criteria [1] in the surgical specimens. In particular, the absence of invasive cancer in both the primary breast tumor and axillary lymph nodes qualified for pCR. Cases of residual in situ cancer only were also considered to be pCR.

In patients who underwent a segmental mastectomy with axillary lymph node dissection with ≤ 3 positive nodes, postoperative irradiation treatment was delivered to residual breast, or in those with >4 positive nodes, delivered to both residual breast and supraclavicular lymph region. In patients who underwent a modified radical mastectomy with >4 positive axillary nodes, postoperative irradiation treatment was delivered to the chest wall and supraclavicular region.

Locoregional radiotherapy was instituted within 8 weeks of the completion of surgery. All patients were followed up regularly by physical and blood examinations with mandatory screening by chest X-ray, ultrasound, computed tomography and bone scan. The median and mean duration of follow-up were 33 and 30 months (range 5–68) for the survivors alive at the date of their last visit ($n = 22$).

All procedures were carried out with the prior informed consent of the patients. This study was approved by the Institutional Review Board and the ethical committee of our hospital (registration #22–217).

Immunohistochemistry

Briefly, 4-μm-thick paraffin sections were dewaxed in xylene, rehydrated through a series of graded alcohols, placed in Target Retrieval Solution (pH 9.0, DakoCytomation, Kyoto, Japan) and submitted to heat retrieval using an autoclave for 30 min. After heating, the slides were allowed to cool to room temperature and endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 5 min. The slides were then incubated overnight at 4°C with the primary antibodies detailed in Table 2. To detect basal cytokeratins, a cytokeratin 5/6/14/17 antibody cocktail was used. Immunohistochemical staining was performed using an Envision Kit (DAKO). The slides were incubated with horseradish peroxidase-labeled polymers? conjugated with secondary antibodies and then with substrate-chromogen (3,3-diaminobenzidine tetrahydrochloride) solution, followed by light counterstaining with Mayer's hematoxylin.

Assessment of immunostaining

All slides were reviewed and scored independently by two of the authors (H.M. and Y.A.) without knowledge of the demographic or treatment response information. Interobserver variations were resolved by re-evaluation and discussion to reach consensus. The cutoff for ER, PgR, CK5/6/14/17, EGFR and c-Kit was 10% positive cells, irrespective of intensity. A HER2-negative result was defined as either HER2 0 or 1+ (strongly positive in <10% of cancer cells); a positive was concluded with HER2 2+ (moderately positive in >10% of cancer cells) and gene amplified <twofold in fluorescence in situ hybridization. Distinct nuclear staining for MGMT, MLH1, MSH2 and BRCA1 was considered to be positive, regardless of the staining intensity. Normal ductal epithelium which exhibited nuclear staining for these proteins was considered as an internal positive control. The percentage of positive tumor cells was assessed and classified into four categories: <10; 10–50; 51–90; >90%.

Table 3 Relationship between clinical and pathological responses to neoadjuvant chemotherapy

	pCR		Non-pCR	
	No.	%	No	%
CR	3	100	0	0
PR	6	37.5	10	62.5
SD	1	12.5	7	87.5
PD	0	0	5	100

pCR pathological complete response, *CR* complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease

$P = 0.015$

Statistical analysis

Categorical analysis of variables was performed using either the Chi-squared test (with Yates' correction) or the Fisher's exact test, as appropriate. Cancer-related survival time was measured from the date of surgery to the end of follow-up or death due to breast cancer or other causes. Recurrence-free survival time was defined as the time from surgery to recurrent disease (alive) or death, with or without recurrence. Survival curves were generated by the Kaplan–Meier method and differences assessed by the log-rank test. A P value of <0.05 was considered statistically significant. The statistical data were obtained using StatView, Version 5.0 (SAS Institute Inc., Cary, NC, USA).

Results

Clinical and pathological responses to neoadjuvant chemotherapy and survival

Regarding clinical responses to NACT, CR was observed in 3 patients (9.4%), PR in 16 (50.0%), SD in 8 (25.0%), and PD in 5 (15.6%). With pathological responses, pCR was obtained in 10 of 32 cases (31%). In the twenty-two non-pCR cases, ten demonstrated 60–99% reduction of primary tumor and twelve <60% reduction. All of the patients with clinical CR featured pCR but none of the patients with PD ($P = 0.015$; Table 3).

Cancer-related ($P = 0.013$) and disease-free ($P = 0.023$) survival rates were significantly higher in the pCR than the non-pCR group. Kaplan–Meier plots illustrating associations of pathological response to NACT with survival are shown in Fig. 1.

Expression of MGMT, MLH1, MSH2 and BRCA-1

The immunohistochemical findings for these proteins are summarized in Table 4. When results were stratified into two groups, i.e., attenuated (≤50%) and normal (>51%), attenuated expression of MGMT, MLH1, MSH2 and BRCA1 was observed in 12/32 (38%), 0/32 (0%), 5/32 (16%) and 28/32 (88%) cases, respectively. The frequencies significantly varied ($P < 0.001$), with no significant relationships to be found. In addition, no significant associations were detected between patient characteristics (patient's age, menopausal status, or clinical and pathological stages) and protein expression (data not shown).

Paired samples consisting of a tru-cut biopsy taken at the time of diagnosis (before NACT) and surgically resected material after NACT were available for 19 of the non-pCR cases. Changes in expression of MGMT, MLH1, MSH2 and BRCA1 during NACT are detailed in Table 5. In the 19 cases, decrease in expression were more frequently observed for MGMT than for MLH1, MSH2 or BRCA1 ($P = 0.021$). Cases with decreased expression in MGMT during NACT (4/9 cases, 44%) frequently tended to show ≥60% tumor reduction, as compared to those with no decrease (2/10, 20%; not statistically significant).

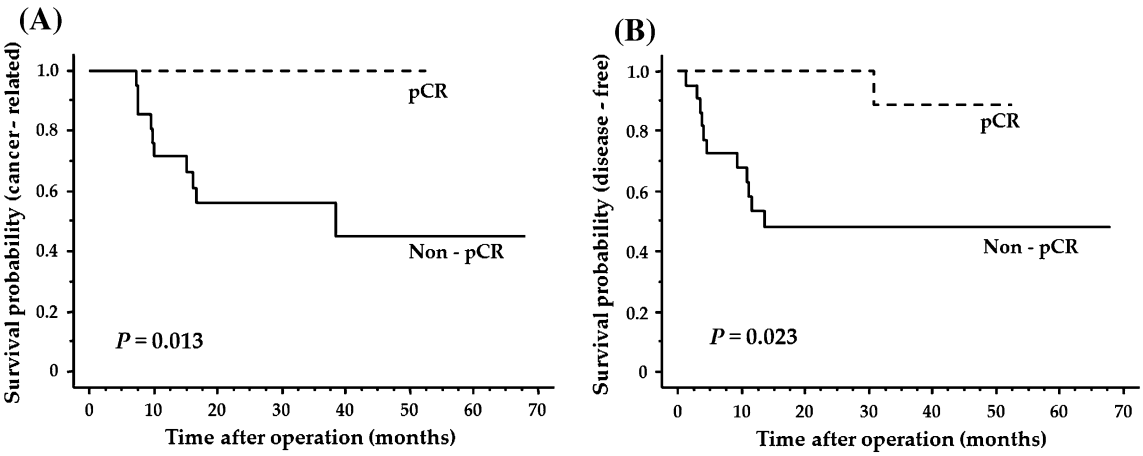

Fig. 1 Cancer-related (**a**) and disease-free (**b**) survival curves with reference to pathological response to neoadjuvant chemotherapy

Table 4 Distribution of expression of MGMT, MLH1, MSH2 and BRCA1 before chemotherapy in biopsy samples

Immunoreactivity	MGMT		MLH1		MSH2		BRCA1		*P* value
	No.	%	No.	%	No.	%	No.	%	
<10%	0	0	0	0	2	6.2	21	65.6	<0.001
10–50%	12	37.5	0	0	3	9.4	7	21.9	
51–90%	6	18.7	5	15.6	12	37.5	4	12.5	
>90%	14	43.8	27	84.4	15	46.9	0	0	
Attenuated (≤50%)	12	37.5	0	0	5	15.6	28	87.5	<0.001
Normal (>50%)	20	62.5	32	100	27	84.4	4	12.5	

Table 5 Change in expression of MGMT, MLH1, MSH2 and BRCA1 before and after neoadjuvant chemotherapy

Change in expression	MGMT		MLH1		MSH2		BRCA1	
	No.	%	No.	%	No.	%	No.	%
Increase	2	10.5	1	5.3	1	5.3	1	5.3
No change	8	42.1	18	94.7	15	78.9	14	73.7
Decrease	9	47.4	0	0	3	15.8	4	21.0

$P = 0.021$

Expression in a representative case (#5) is illustrated in Fig. 2.

Patients' characteristics and immunohistochemistry and their relationship with pCR

On evaluation of associations among pCR, patients' characteristics and immunohistochemical patterns, only attenuated expression of MGMT was found to be significantly predictive of a pCR ($P = 0.018$; Table 6).

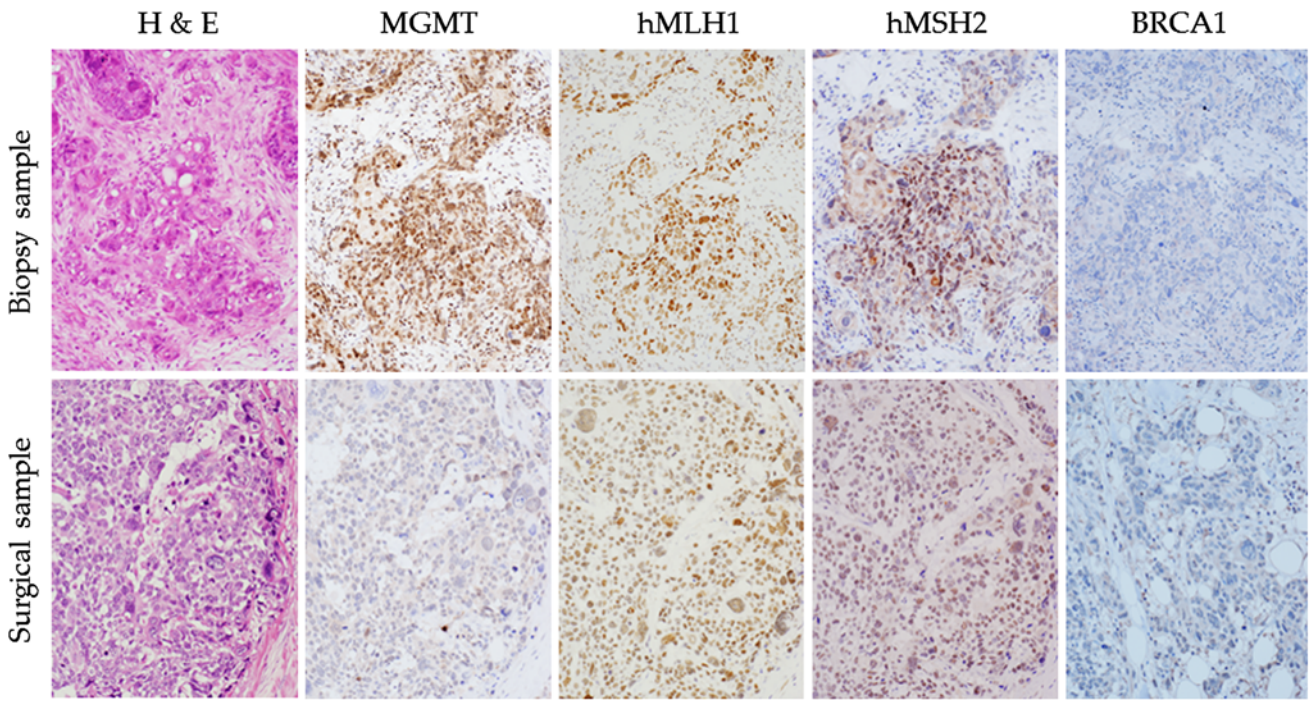

Fig. 2 H&E staining and immunohistochemistry of serial sections of paired samples obtained from a biopsy (original magnification, ×70) and surgical material (original magnification, ×82) in non-pCR from case No. 5. BLBC, shown as expansive and medullary growth pattern, consists of tumor cells with high nuclear grade and pleomorphism. Note marked decrease of MGMT expression; 50–90% nuclear immunopositivity for MGMT in tumor cells in a tru-cut biopsy sample before neoadjuvant chemotherapy and <10% nuclear immunopositivity in tumor cells in the surgically resected sample after neoadjuvant chemotherapy. No change is apparent in immunopositivity for MLH1 (>90% nuclear expression), MSH2 (50–90% nuclear immunopositivity) or BRCA1 (<10% nuclear expression) expression before and after neoadjuvant chemotherapy

Table 6 Univariate analysis of patient characteristics and expression of MGMT, MLH1, MSH2 or BRCA1 associated with pCR versus non-pCR to neoadjuvant chemotherapy

Characteristics	pCR		Non-pCR		*P* value
	No.	%	No.	%	
Total patients	10	31.3	22	68.7	
Age, years					
≤50	4	30.8	9	69.2	NS
>50	6	31.6	13	68.4	
Menopausal status					
Premenopausal	5	41.7	7	58.3	NS
Postmenopausal	5	25.0	15	75.0	
Initial tumor size					
T1 or T2	8	33.3	16	66.7	NS
T3 or T4	2	25.0	6	75.0	
Initial lymph node status					
N0 or N1	8	29.6	19	70.4	NS
N2 or N3	2	40.0	3	60.0	
Initial clinical stage					
II	8	32.0	17	68.0	NS
III	2	28.6	5	71.4	
MGMT expression					
Attenuated	7	58.3	5	41.7	0.018
Normal	3	15.0	17	85.0	
MLH1 expression					
Attenuated	0	0	0	0	NS
Normal	10	31.3	22	68.7	
MSH2 expression					
Attenuated	3	60.0	2	40.0	NS
Normal	7	25.9	20	74.1	
BRCA1 expression					
Attenuated	10	35.7	18	64.3	NS
Normal	0	0	4	100	

Discussion

Regardless of the breast cancer subtype, pCR is a powerful indicator of prolonged survival in patients receiving NACT [1, 2]. We therefore tested survival impact of pathological response to NACT especially in BLBCs reported to be more sensitive to NACT than luminal lesion [8, 9]. In the present study, the pCR rate in BLBC was 31%, consistent with the previous reports stated 21–45% [9, 25, 26]. We also found a significant benefit of pCR on survival in 32 patients with BLBC, in line with earlier finding that none of 34 patients with pCR to NACT relapsed or died [8]. Conversely, two other studies failed to show statistically significant differences between pCR and non-pCR groups with 50 and 22 cases of BLBC [25, 26]. The reasons remain unclear and point to the necessity for larger scale comprehensive studies.

Since our patients with BLBC who achieved pCR showed improved prognosis, the current study was undertaken to determine whether the expression of MGMT, MLH1, MSH2 and BRCA1 could affect the pCR to NACT. Although patients' characteristics and TNM classification were not significantly predictive of a pCR, attenuated expression of MGMT before NACT did serve as a significant predictor. In contrast, MGMT gene expression measured by reverse transcription-PCR was not predictive of response to neoadjuvant chemotherapy [27]. MGMT immunoreactivity has been reported to be correlated with local recurrence, distant metastasis and prognosis in breast carcinoma [28, 29]. To the best of our knowledge, there is no report of relationship between MGMT immunoreactivity and response to neoadjuvant chemotherapy.

As a result of defective MGMT function through promoter methylation, evidence has been provided for prediction of benefit from alkylating agent therapy in glioma [30] and glioblastoma [31], and from multidrug regimens with cyclophosphamide for B-cell lymphomas [32]. In an experimental study, mice overexpressing MGMT proved more resistant to carcinogenesis induced by alkylating agents, whereas knock-out mice were more sensitive [33]. Methylating agents could be shown to effectively inactivate MGMT in human breast carcinoma cells and xenografts, resulting in substantial increase in sensitivity to growth inhibition by alkylating agent [34]. Furthermore, acquisition of doxorubicin resistance by a human breast carcinoma cell line was associated with MGMT hypomethylation of the promoter region [35]. Interestingly, the current study demonstrated that NACT resulted in decreased expression of MGMT in 40% of cases. Moreover, decreased expression in MGMT during NACT enhanced tumor reduction. In chemosensitive BLBC, NACT might selectively alter MGMT expression through mechanisms including promoter methylation.

An attenuated expression of MLH1 and MSH2 may preferentially occur in breast carcinomas with a poorer differentiation [16], but our frequencies of inactivation for these proteins were low at 0 and 16%. In sporadic breast cancer, approximately 20–40% exhibited decreased expression of these proteins [14–16]. In line with our results, no significant correlation was identified between MLH1 and MSH2 with age, tumor size and lymph node metastasis [16]. Current study is discordant with some observations that attenuated MLH1 expression is closely associated with results of NACT [13, 15]. Absent or reduced BRCA1 expression has been observed in approximately 80% of BLBCs, linked with advanced lymph node stage, large size and vascular invasion [36]. In a study of sporadic breast

cancer patients, those with low levels of BRCA1 mRNA expression attained better response to anthracycline-based chemotherapy [37]. Furthermore, the presence of BRCA1-negative foci in biopsy specimens before neoadjuvant epirubicin plus cyclophosphamide treatment was inversely correlated with tumor response [38]. However, there is conflicting evidence as to whether tumors with inactivation of BRCA1 demonstrate particular clinical or pathological features or obtain greater benefit from DNA-damage-based chemotherapy, in line with our findings.

Immunohistochemistry, which is the most practical method for assessing protein expression changes, not only provides a semiquantitative assessment of protein abundance but also defines the cellular localization. Because no special processing of tissue samples is needed and labor-intensive and expensive diagnostic techniques are avoided, immunohistochemistry is perhaps the most readily adaptable technique to clinical practice. Interobserver reproducibility of immunohistochemical scoring is sometimes problematic. However, interobserver variations were easily resolved by re-evaluation and discussion to reach consensus because MGMT immunostaining is distinct and reliable in the present study as well as previous reports [28, 29]. In this study, patients with attenuated MGMT expression in their lesions had a 2.3 times higher probability of achieving a pCR than those with preserved expression. In our limited study, MGMT status might be a predictive factor for pCR to neoadjuvant anthracycline-based plus taxane combination chemotherapy, and a potential aid to selection of appropriate NACT for Japanese patients with BLBC.

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Conflict of interest The authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

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