Preoperative systemic chemotherapy, initially used only for inflammatory and inoperable locally advanced breast cancer, has recently been more widely used for operable disease (Kaufmann *et al*, 2006; Gralow *et al*, 2008). Although systemic chemotherapy before and after surgery results in identical survival rates, preoperative chemotherapy has the advantages of eliciting a tumour response in individual patients and increasing the number of patients eligible for breast-conserving surgery (Kaufmann *et al*, 2006; Gralow *et al*, 2008). The residual tumour burden in the breast and axillary lymph node after preoperative chemotherapy is the strongest prognostic factor (Carey *et al*, 2005; von Minckwitz *et al*, 2012). Moreover, the residual tumour burden in the axillary node is a better prognostic factor than the response of the primary tumour (Rouzier *et al*, 2002; von Minckwitz *et al*, 2012), and residual micrometastatic disease is predictive of poor prognosis (Fisher *et al*, 2002; Klauber-DeMore *et al*, 2006; Sakakibara *et al*, 2009). Thus, accurate evaluation of axillary node status is of great clinical significance in patients with breast cancer who are treated with preoperative chemotherapy.

Conventional pathological examination of lymph nodes has three potential limitations that affect the accurate and reproducible measurement of the total residual metastatic volume. First, pathological examinations only partially evaluate each node, and this may lead to underestimation of the nodal status. Although underestimation of the metastasis volume can be reduced by serial sectioning, this imposes a heavy workload for pathologists. Second, histological changes caused by chemotherapy, such as decreases in cellularity with stromal fibrosis, aggregates of foamy histiocytes, and degenerated cancer cells, can affect tumour burden assessment (Sahoo and Lester, 2009). Finally, the practice of pathological evaluation of lymph nodes is not standardised. Examination protocols vary from one institution to another (Cserni *et al*, 2004), and there is inter-observer variability in diagnosing the tumour burden, particularly for low-volume metastasis (Cserni *et al*, 2008).

The one-step nucleic acid amplification (OSNA) assay is a novel molecular method for the lymph node staging of breast cancer (Tsujimoto *et al*, 2007) that has been tested in multiple series (Cserni, 2012; Tamaki, 2012). The results of this semi-automated molecular assay based on the quantification of cytokeratin 19 (CK19) mRNA display a 96% concordance rate with detailed pathology complemented by immunohistochemistry when alternate slices of the same lymph node are used for the two tests (Cserni, 2012; Tamaki, 2012). The OSNA assay is accepted and routinely used in >230 institutions in Spain, Japan, Italy, the UK, France, and other countries (http://lifescience.sysmex.co.jp/ls/products/osna/index.html). However, the performance of the OSNA assay has not been evaluated in patients treated with preoperative systemic therapy. This assay can potentially contribute to the accurate, reproducible, and standardised evaluation of the lymph node status after systemic therapy. In this prospective multicentre trial, we compared the performance of the OSNA assay with that of pathological examination and investigated the effect of chemotherapy-induced histological changes on its performance.

MATERIALS AND METHODS

Enrolled patients and lymph nodes. Axillary lymph nodes were obtained from patients with breast cancer who underwent standard preoperative chemotherapy followed by axillary lymph node dissection between May 2010 and March 2011 at one of three Japanese institutions. This study was approved by the ethics committee of each institution. Patients were given the necessary written information about the study, and only the lymph nodes from patients who gave their consent were included in the analysis.

A maximum of four lymph nodes sampled from the level-I axillary region were included for a single patient. The remaining nodes were evaluated by permanent histology using single-sectioned nodes. The clinical and pathological TNM classification and staging and the level of axillary lymph node dissection of each patient were classified according to the seventh edition of the American Joint Committee on Cancer Staging Manual (Edge *et al*, 2010).

Lymph node examination process. Sampled fresh lymph nodes larger than 4 mm in short axis were immediately sliced using the cutting device developed by Tsujimoto *et al* (2007), resulting in two 2-mm central sections (i.e., sections b and c) with additional two excess sections on both sides (i.e., sections a and d; Figure 1A). Alternate pieces (i.e., sections a and c) were evaluated by two-level pathological examination, and the others (i.e., sections b and d) were examined using the OSNA assay. Lymph nodes sized 4 mm or less in short axis were cut by the cutting device into two pieces (Figure 1B). One piece (i.e., section a') was evaluated by pathological examination, and the other piece (i.e., section b') was examined using the OSNA assay. The lymph node slices for pathological examination were fixed with formalin and embedded in paraffin, and the slices for the OSNA assay were stored frozen at −80 °C until measurement.

Pathological examination. A pair of 4-μm-thick sections was prepared from each slice (Figure 1): one section was stained with haematoxylin-and-eosin and the other was immunostained with a CK19 antibody (Clone RCK108; Dako, Glostrup, Denmark). All slides were centrally reviewed by one experienced pathologist (RH) who was blinded to the results of the OSNA assay. Each node was classified as having macrometastasis (>2.0 mm in size), micrometastasis (>0.2–2.0 mm in size), isolated tumour cells (ITC, ⩽0.2 mm in size), or no cancer cells according to the seventh edition of the American Joint Committee on Cancer classification (Edge *et al*, 2010). Macrometastasis and micrometastasis were regarded as positive findings, and ITCs and no cancer cells were regarded as negative findings. Furthermore, stromal fibrosis, aggregates of foamy histiocytes, and degenerated cancer cells in lymph nodes were regarded as chemotherapy-induced histological changes.

The OSNA assay. The procedure for the OSNA assay has been previously described in detail (Tsujimoto *et al*, 2007). Briefly, frozen slices of lymph nodes were homogenised with 4 ml of lysis buffer solution (Lynorhag; Sysmex Corporation, Kobe, Japan) and

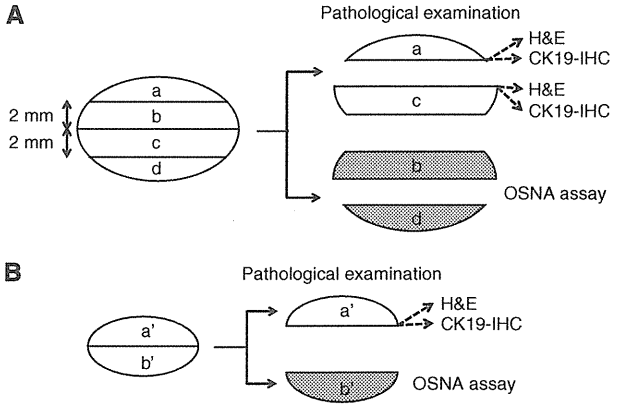

Figure 1. Lymph node examination process. Lymph nodes larger than 4 mm were sliced into four pieces (**A**), and lymph nodes sized 4 mm or less were cut into two pieces (**B**). H&E, haematoxylin-and-eosin staining; CK19-IHC, cytokeratin 19 immunohistochemistry; OSNA, one-step nucleic acid amplification.

centrifuged at 10 000 **g** at room temperature. Two microlitres of supernatant was analysed using the RD-100*i* System (Sysmex Corporation), an automated molecular detection system that uses a reverse transcription loop-mediated isothermal amplification method (Notomi *et al*, 2000), and the LynoampBC Kit (Sysmex Corporation). The degree of amplification was determined on the basis of a reaction by-product, pyrophosphate (Mori *et al*, 2001). The resultant change in turbidity on the precipitation of magnesium pyrophosphate was then correlated with the CK19 mRNA copy number per microlitre of the original lysate via a standard curve established beforehand using three calibrators containing different CK19 mRNA copy numbers. The number of CK19 mRNA copies per microlitre was extrapolated from the standard curve for both the measurement sample and a 1 : 10 diluted sample. The cutoffs for negative/positive results and (+)/(++) were set at 250 and 5000 copies per microlitre, respectively (Tsujimoto *et al*, 2007). Positive (+) was considered equivalent to micrometastasis (not including ITC), and positive (++) was considered equivalent to macrometastasis (Tsujimoto *et al*, 2007). In situations in which the reaction was inhibited in the measurement sample, the copy numbers in the diluted sample were used.

Additional investigation of discordant lymph nodes. For lymph nodes that were positive on the OSNA assay and negative (no cancer cells) on pathological examination, the paraffin blocks of the lymph node tissue were step-sectioned with 0.2-mm intervals until the tissue was exhausted. At each level, two microscopic slides were made: one was used for haematoxylin-and-eosin staining and the other was used for CK19 immunostaining. All stained slides were microscopically examined by the central pathologist (RH).

Statistical analysis. With the result of the pathological examination as the gold standard, the accuracy, sensitivity, and specificity of the OSNA assay were calculated. The differences in the accuracy, sensitivity, and specificity between lymph nodes with and without chemotherapy-induced histological changes were assessed by the two-population *z*-test. Confidence intervals (CIs) were set at the 95% level. *P*-values of <0.05 were considered statistically significant. All statistical analyses were performed using R statistical software (version 2.10.1, http://www.r-project.org/; Ihaka and Gentleman, 1996).

RESULTS

Enrolled patients and lymph nodes. In total, 307 lymph nodes obtained from 80 patients who underwent surgery after preoperative chemotherapy were included in the study. Of these, four nodes were excluded because of a lack of lymph node tissue, and one node was excluded because it was not subjected to the OSNA assay. Thus, 302 nodes from 80 patients were included in the analysis. The characteristics of the 80 patients are shown in Table 1. Of the 80 patients, 71 (88.8%) were diagnosed with node-positive before receiving chemotherapy. Of the 71 patients, 8, 52, and 11 were confirmed as positive by sentinel node biopsy, fine needle aspiration cytology, and clinical examination/imaging, respectively. The mean and median numbers of enrolled lymph nodes included from a single patient were 3.8 and 4, respectively. Of the 302 nodes, 192 nodes (63.6%) were sliced into four pieces, and 110 nodes (36.4%) were cut into two pieces.

Overall performance of the OSNA assay. The results of the pathological examination and the OSNA assay were concordant for 275 of 302 nodes (accuracy, 91.1%; 95% CI, 87.3–94.0%; Table 2). Of the 60 nodes identified as positive for metastasis on pathological examination, 53 nodes were identified as positive on the OSNA

Table 1. Patient characteristics

Characteristics	No.	%
No. of patients	80	100.0%
Age (years)		
Median (range)	52 (30–71)	
Clinical N status before chemotherapy		
cN0	9	11.3%
cN1	59	73.8%
cN2	2	2.5%
cN3	10	12.5%
Clinical stage before chemotherapy		
IIA	15	18.8%
IIB	39	48.8%
IIIA	12	15.0%
IIIB	3	3.8%
IIIC	9	11.3%
IV	2	2.5%
Histological type		
Invasive ductal	78	97.5%
Invasive micropapillary	2	2.5%
Oestrogen receptor status		
−	25	31.3%
+	55	68.8%
Progesterone receptor status		
−	38	47.5%
+	42	52.5%
HER2 status		
−	54	67.5%
+	18	22.5%
Uncertain	8	10.0%
Chemotherapy regimen		
Anthracycline	4	5.0%
Anthracycline/taxane	50	62.5%
Anthracycline/taxane/carboplatin	3	3.8%
Anthracycline/taxane/trastuzumab	15	18.8%
Taxane	7	8.8%
Taxane/trastuzumab	1	1.3%
Breast surgery		
Total mastectomy	47	58.8%
Partial mastectomy	33	41.3%
Axillary lymph node dissection		
Level I	1	1.3%
Level II	70	87.5%
Level III	9	11.3%
No. of lymph nodes removed		
Median (range)	16 (5–38)	
Pathological T status after chemotherapy		
ypT0	8	10.0%
ypTis	10	12.5%
ypT1	21	26.3%
ypT2	20	25.0%
ypT3	21	26.3%
Pathological N status after chemotherapy		
ypN0	32	40.0%
ypN1	35	43.8%
ypN2	7	8.8%
ypN3	6	7.5%

Abbreviation: HER2 = human epidermal growth factor receptor-2.

assay (sensitivity, 88.3%; 95% CI, 77.4–95.2%). Of the 242 nodes identified as negative for metastasis on pathological examination, 222 nodes were identified as negative on the OSNA assay (specificity, 91.7%; 95% CI, 87.5–94.9%).

Chemotherapy-induced histological changes and performance of the OSNA assay. Of the 302 lymph nodes, 66 (21.9%) displayed chemotherapy-induced histological changes. The accuracy, sensitivity, and specificity of the OSNA assay relative to the reference pathology were 90.9% (60 out of 66), 88.9% (32 out of 36), and 93.3% (28 out of 30), respectively, among lymph nodes with chemotherapy-induced histological changes and 91.1% (215 out of 236), 87.5% (21 out of 24), and 91.5% (194 out of 212), respectively, among lymph nodes without histological changes (Table 3). There were no differences in accuracy, sensitivity, or specificity between the two groups ($P = 0.96$, 0.87, and 0.73, respectively).

Lymph nodes with discordant results. Of the 302 lymph nodes, 27 (8.9%) showed discordant results between the pathological examination and the OSNA assay (Table 4). Of these 27 nodes, 20 were negative on pathological examination and positive on the OSNA assay (false positive when using pathology as the gold standard), whereas 7 were positive on pathological examination and negative on the OSNA assay (false negative when using pathology as the gold standard).

Of the 20 nodes with false-positive results, ITCs were identified in five nodes during the original pathological assessment. Moreover, cancer cells were identified in two nodes (one with micrometastasis and one with ITC) during the additional pathological assessment. In contrast, no cancer cells were identified in 13 nodes during the additional pathological examination; the median CK19 mRNA copy number was 450 (range, 280–250 000).

Of the seven nodes with false-negative results, six nodes displayed micrometastasis and one node exhibited macrometastasis. The median size of metastasis on pathology was 0.8 mm (range, >0.2–12.0 mm). In all seven nodes, CK19 protein expression was detected by immunohistochemistry.

Table 2. Comparison of the results of the OSNA assay with pathological examination

	Pathology			
	Positive		Negative	
	Macro	Micro	ITC	None
OSNA				
Positive				
(+ +)	32	3	2	4
(+)	10	8	3	11
Negative	1	6	1	221

Abbreviations: ITC = isolated tumour cells; OSNA = one-step nucleic acid amplification.

DISCUSSION

To the best of our knowledge, this prospective multicentre trial is the first study to evaluate the performance of a molecular assay in detecting lymph node metastasis in patients with breast cancer who were treated with preoperative systemic therapy. The OSNA assay can detect the residual tumour burden in lymph nodes after chemotherapy as accurately as conventional pathology. The overall performance of the OSNA assay in this study is almost equivalent to the results of two pooled analyses of previous trials in which similar protocols were used in patients who did not receive preoperative systemic therapy (accuracy, 93.6–96.1%; sensitivity, 87.9–91.7%; specificity, 94.8–97.0%; Cserni, 2012; Tamaki, 2012). Moreover, chemotherapy-induced histological changes did not affect the performance of the OSNA assay. The performance of the assay for lymph nodes with chemotherapy-induced histological changes was similar to that for lymph nodes without histological changes as well as that reported in the aforementioned pooled analyses.

The main reason for the discordant results between the OSNA assay and conventional pathology may be tissue allocation bias. As per the protocol of this study, small metastases localised in only one slice inevitably result in discordant findings. Of the 20 nodes displaying false-positive results, 7 nodes showed cancer cells on the original or additional pathological assessment slides. In addition, 10 nodes had a low tumour burden of no >1000 copies. Thus, in

Table 3. Chemotherapy-induced histological changes and performance of the OSNA assay

	Pathology							
	Presence of histological changes ($n = 66$)				Absence of histological changes ($n = 236$)			
	Positive		Negative		Positive		Negative	
	Macro	Micro	ITC	None	Macro	Micro	ITC	None
OSNA								
Positive								
(+ +)	17	1	1	0	15	2	1	4
(+)	8	6	1	0	2	2	2	11
Negative	0	4	0	28	1	2	1	193
Accuracy (95% CI)	90.9% (0.81–0.97)				91.1% (0.87–0.94)			
Sensitivity (95% CI)	88.9% (0.74–0.97)				87.5% (0.68–0.97)			
Specificity (95% CI)	93.3% (0.78–0.99)				91.5% (0.87–0.95)			

Abbreviations: CI = confidence interval; ITC = isolated tumour cells; OSNA = one-step nucleic acid amplification.

Table 4. Lymph nodes with discordant results and the possible cause

	OSNA		Pathology			
Lymph node	Result	CK19 mRNA (copy μl^{-1})	Original assessment (size, mm)	Additional assessment (size, mm)	CK19 protein	Possible cause
False positive						
JC28-4	(+ +)	32 000	ITC (≤0.2)	NA	(+)	Allocation bias
SL07-4	(+ +)	6300	ITC (≤0.2)	NA	(+)	Allocation bias
CR02-3	(+)	2300	ITC (≤0.2)	NA	(+)	Allocation bias
JC07-4	(+)	460	ITC (≤0.2)	NA	(+)	Allocation bias
JC26-4	(+)	300	ITC (≤0.2)	NA	(+)	Allocation bias
SL02-4	(+)	280	None	Micro (0.8)	(+)	Allocation bias
CR02-2	(+ +)	13 000	None	ITC (≤0.2)	(+)	Allocation bias
SL07-2	(+ +)	250 000	None	None	NA	Human error
SL12-4	(+ +)	6300[a]	None	None	NA	Allocation bias
CR17-4	(+ +)	5600	None	None	NA	Allocation bias
JC11-4	(+)	1000	None	None	NA	Allocation bias
CR07-2	(+)	960	None	None	NA	Allocation bias
JC18-1	(+)	710[a]	None	None	NA	Allocation bias
SL06-4	(+)	450[a]	None	None	NA	Allocation bias
CR13-3	(+)	410	None	None	NA	Allocation bias
JC10-2	(+)	400	None	None	NA	Allocation bias
SL09-1	(+)	400	None	None	NA	Allocation bias
SL14-1	(+)	330	None	None	NA	Allocation bias
SL06-1	(+)	300	None	None	NA	Allocation bias
SL13-2	(+)	280	None	None	NA	Allocation bias
False negative						
SL07-3	(−)	<250	Macro (12.0)	NA	(+)	Human error
JC19-2	(−)	ND	Micro (1.0)	NA	(+)	Allocation bias
JC35-2	(−)	ND	Micro (1.0)	NA	(+)	Allocation bias
SL09-4	(−)	<250	Micro (0.8)	NA	(+)	Allocation bias
JC21-1	(−)	ND	Micro (0.8)	NA	(+)	Allocation bias
JC04-3	(−)	<250	Micro (0.5)	NA	(+)	Allocation bias
JC21-3	(−)	ND	Micro (0.2[b])	NA	(+)	Allocation bias

Abbreviations: CK19 = cytokeratin 19; ITC = isolated tumour cells; NA = not available; ND = not detected; OSNA = one-step nucleic acid amplification.
[a]CK19 mRNA copy numbers in the diluted sample.
[b]Just over 0.2 mm in size.

these 17 nodes, tissue allocation bias could have resulted in discordant findings. In addition, two nodes (#SL12-4 and #CR17-4) had metastasis with 5000–6000 copies; these copy numbers suggest that the tumours are approximately 2 mm in size (Tsujimoto *et al*, 2007). Although the metastatic status of these two nodes is indeterminate, tissue allocation bias is also suspected as the cause of the discordant results. Furthermore, all seven nodes with false-negative results were positive for CK19 protein expression. Although the OSNA assay may miss metastases that do not express CK19 mRNA in principle, the false-negative results in this study did not appear to be caused by the absence or low expression of CK19 mRNA. Of the seven nodes, six had micrometastasis of ⩽1.0 mm in size. Therefore, tissue allocation bias is a possible cause of the discordant results for these six nodes.

The discordant results of the remaining two nodes (#SL07-2 and #SL07-3) may be due to human error. In lymph node #SL07-2, the CK19 mRNA copy number was high, but no cancer cells were detected during the original or additional pathological examination. In contrast, in lymph node #SL07-3, the CK19 mRNA copy number was low, whereas a large metastatic lesion expressing CK19 was observed during the pathological examination. Lymph nodes #SL07-2 and #SL07-3 were sampled from the same patient. Therefore, the pieces for the OSNA assay or the pathological samples of the two nodes may have been switched during the handling of the samples.

In clinical practice, the OSNA assay can contribute to the accurate, reproducible, and standardised evaluation of the residual tumour burden after preoperative chemotherapy. When a whole lymph node or a large amount of a node is examined using the OSNA assay, more micrometastases can be detected than by the use of routine pathological examinations (Osako *et al*, 2011a, b, 2012; Remoundos *et al*, 2013). This is reasonable considering that routine pathology analyses only limited a part of the lymph node, whereas the OSNA assay can thoroughly evaluate the entire lymph node. Patients with negative nodes or micrometastases who were not treated with preoperative chemotherapy had identical survival rates, whereas the survival rate of patients with micrometastases in lymph nodes after chemotherapy was similar to that of patients with macrometastases and significantly worse than that of patients with negative nodes (Fisher *et al*, 2002). Thus, the OSNA assay facilitates prediction of the prognosis of patients treated with preoperative chemotherapy more accurately than conventional pathological examinations. Although further chemotherapy may potentially not be delivered after neoadjuvant chemotherapy plus surgery, adjuvant therapies including radiation, hormone, and molecular-target therapies can be considered for these patients. Therefore, this more accurate diagnosis of lymph node status can enable to personalise the adjuvant therapy for each of the patients.

In conclusion, the OSNA assay can detect residual tumour burden in lymph nodes after chemotherapy as accurately as

conventional pathology even when chemotherapy-induced histological changes are present. The main cause of discordant results may be tissue allocation bias. Therefore, the OSNA assay can contribute to the accurate, reproducible, and standardised evaluation of lymph node status after preoperative chemotherapy.

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REFERENCES

Carey LA, Metzger R, Dees EC, Collichio F, Sartor CI, Ollila DW, Klauber-DeMore N, Halle J, Sawyer L, Moore DT, Graham ML (2005) American Joint Committee on Cancer tumor-node-metastasis stage after neoadjuvant chemotherapy and breast cancer outcome. *J Natl Cancer Inst* **97**(15): 1137–1142.

Cserni G (2012) Intraoperative analysis of sentinel lymph nodes in breast cancer by one-step nucleic acid amplification. *J Clin Pathol* **65**(3): 193–199.

Cserni G, Amendoeira I, Apostolikas N, Bellocq JP, Bianchi S, Boecker W, Borisch B, Connolly CE, Decker T, Dervan P, Drijkoningen M, Ellis IO, Elston CW, Eusebi V, Faverly D, Heikkila P, Holland R, Kerner H, Kulka J, Jacquemier J, Lacerda M, Martinez-Penuela J, De Miguel C, Peterse JL, Rank F, Regitnig P, Reiner A, Sapino A, Sigal-Zafrani B, Tanous AM, Thorstenson S, Zozaya E, Fejes G, Wells CA (2004) Discrepancies in current practice of pathological evaluation of sentinel lymph nodes in breast cancer. Results of a questionnaire based survey by the European Working Group for Breast Screening Pathology. *J Clin Pathol* **57**(7): 695–701.

Cserni G, Bianchi S, Vezzosi V, van Diest P, van Deurzen C, Sejben I, Regitnig P, Asslaber M, Foschini MP, Sapino A, Castellano I, Callagy G, Arkoumani E, Kulka J, Wells CA (2008) Variations in sentinel node isolated tumour cells/micrometastasis and non-sentinel node involvement rates according to different interpretations of the TNM definitions. *Eur J Cancer* **44**(15): 2185–2191.

Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A (2010) *American Joint Committee on Cancer Cancer Staging Manual*, 7th edn. Springer: New York, NY, USA.

Fisher ER, Wang J, Bryant J, Fisher B, Mamounas E, Wolmark N (2002) Pathobiology of preoperative chemotherapy: findings from the National Surgical Adjuvant Breast and Bowel (NSABP) protocol B-18. *Cancer* **95**(4): 681–695.

Gralow JR, Burstein HJ, Wood W, Hortobagyi GN, Gianni L, von Minckwitz G, Buzdar AU, Smith IE, Symmans WF, Singh B, Winer EP (2008) Preoperative therapy in invasive breast cancer: pathologic assessment and systemic therapy issues in operable disease. *J Clin Oncol* **26**(5): 814–819.

Ihaka R, Gentleman R (1996) R: a language for data analysis and graphics. *J Comp Graph Stat* **5**: 299–314.

Kaufmann M, Hortobagyi GN, Goldhirsch A, Scholl S, Makris A, Valagussa P, Blohmer JU, Eiermann W, Jackesz R, Jonat W, Lebeau A, Loibl S, Miller W, Seeber S, Semiglazov V, Smith R, Souchon R, Stearns V, Untch M, von Minckwitz G (2006) Recommendations from an international expert panel on the use of neoadjuvant (primary) systemic treatment of operable breast cancer: an update. *J Clin Oncol* **24**(12): 1940–1949.

Klauber-DeMore N, Ollila DW, Moore DT, Livasy C, Calvo BF, Kim HJ, Dees EC, Sartor CI, Sawyer LR, Graham 2nd M, Carey LA (2006) Size of residual lymph node metastasis after neoadjuvant chemotherapy in locally advanced breast cancer patients is prognostic. *Ann Surg Oncol* **13**(5): 685–691.

Mori Y, Nagamine K, Tomita N, Notomi T (2001) Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochem Biophys Res Commun* **289**(1): 150–154.

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* **28**(12): E63.

Osako T, Iwase T, Kimura K, Masumura K, Horii R, Akiyama F (2012) Incidence and possible pathogenesis of sentinel node micrometastases in ductal carcinoma in situ of the breast detected using molecular whole lymph node assay. *Br J Cancer* **106**(10): 1675–1681.

Osako T, Iwase T, Kimura K, Yamashita K, Horii R, Akiyama F (2011a) Accurate staging of axillary lymph nodes from breast cancer patients using a novel molecular method. *Br J Cancer* **105**(8): 1197–1202.

Osako T, Iwase T, Kimura K, Yamashita K, Horii R, Yanagisawa A, Akiyama F (2011b) Intraoperative molecular assay for sentinel lymph node metastases in early stage breast cancer: a comparative analysis between one-step nucleic acid amplification whole node assay and routine frozen section histology. *Cancer* **117**(19): 4365–4374.

Remoundos DD, Ng VV, Wilson HA, Ahmed F, Chia Y, Cunnick GH (2013) The use of one step nucleic-acid amplification (OSNA) in clinical practice: a single-centre study. *Breast* **22**(2): 162–167.

Rouzier R, Extra JM, Klijanienko J, Falcou MC, Asselain B, Vincent-Salomon A, Vielh P, Bourstyn E (2002) Incidence and prognostic significance of complete axillary downstaging after primary chemotherapy in breast cancer patients with T1 to T3 tumors and cytologically proven axillary metastatic lymph nodes. *J Clin Oncol* **20**(5): 1304–1310.

Sahoo S, Lester SC (2009) Pathology of breast carcinomas after neoadjuvant chemotherapy: an overview with recommendations on specimen processing and reporting. *Arch Pathol Lab Med* **133**(4): 633–642.

Sakakibara M, Nagashima T, Kadowaki M, Onai Y, Fujimori T, Yokomizo J, Suzuki H, Fushimi K, Nakatani Y, Miyazaki M (2009) Clinical significance of axillary microresiduals after neoadjuvant chemotherapy in breast cancer patients with cytologically proven metastases. *Ann Surg Oncol* **16**(9): 2470–2478.

Tamaki Y (2012) One-step nucleic acid amplification assay (OSNA) for sentinel lymph node biopsy. *Breast Cancer;* e-pub ahead of print 9 August 2012; doi:10.1007/s12282-012-0390-x.

Tsujimoto M, Nakabayashi K, Yoshidome K, Kaneko T, Iwase T, Akiyama F, Kato Y, Tsuda H, Ueda S, Sato K, Tamaki Y, Noguchi S, Kataoka TR, Nakajima H, Komoike Y, Inaji H, Tsugawa K, Suzuki K, Nakamura S, Daitoh M, Otomo Y, Matsuura N (2007) One-step nucleic acid amplification for intraoperative detection of lymph node metastasis in breast cancer patients. *Clin Cancer Res* **13**(16): 4807–4816.

von Minckwitz G, Untch M, Blohmer JU, Costa SD, Eidtmann H, Fasching PA, Gerber B, Eiermann W, Hilfrich J, Huober J, Jackisch C, Kaufmann M, Konecny GE, Denkert C, Nekljudova V, Mehta K, Loibl S (2012) Definition and impact of pathologic complete response on prognosis after neoadjuvant chemotherapy in various intrinsic breast cancer subtypes. *J Clin Oncol* **30**(15): 1796–1804.

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ORIGINAL ARTICLE - BREAST ONCOLOGY

Comparison of the Indocyanine Green Fluorescence and Blue Dye Methods in Detection of Sentinel Lymph Nodes in Early-stage Breast Cancer

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ABSTRACT

Purpose. To assess the diagnostic performance of sentinel lymph node (SLN) biopsy using the indocyanine green (ICG) fluorescence method compared with that using the blue dye method, a prospective multicenter study was performed.

Methods. Patients with T1–3 primary breast cancer without clinical lymph node involvement were included in this study. ICG as a fluorescence-emitting source and indigo carmine as blue dye were injected into the subareolar area. Extracted lymph nodes were examined to identify the first, second, and other SLNs. The identified nodes were classified according to the ICG fluorescence signal and blue dye uptake.

Results. Ninety-nine eligible patients were included in this study. The ICG fluorescence method identified an average of 3.4 SLNs (range, 1–8) in 98 of 99 patients (detection rate, 99 %). The number of lymph nodes identified by the fluorescence method was significantly higher than that identified by the blue dye method ($p < 0.001$). SLN involvement was identified in 20 % (20 of 99) of patients, all of whom tested positive for the first SLN. In 16 patients, complete axillary lymph node dissection (ALND) was performed. In 25 % (4 of 16) of these patients, axillary metastases were identified; however, no axillary involvement was found in 8 patients with only one involved node, which was isolated as the first SLN.

Conclusions. High rate of SLN detection was achieved using the ICG fluorescence method. The first SLN identified by fluorescence imaging provides an exact indication of the axillary status. Therefore, the ICG fluorescence method provides precise information required to avoid unnecessary ALND.

For many years, axillary lymph node dissection (ALND) has been performed for prevention of lymph node metastasis in patients with breast cancer. However, ALND is associated with a relatively high risk of complications such as edema of the arms (lymphedema), dyskinesia, and pain, which lower quality of life.[1–3] In the 1990s, sentinel lymph node (SLN) biopsy was proposed for the assessment of axillary lymph node involvement to circumvent unnecessary ALND.

Sentinel lymph node is defined as a lymph node that receives lymph flow directly from the primary tumor. Because this concept was first applied to melanoma patients in 1992, SLN biopsy has become a standard method for evaluating the axillary lymph node status in patients with early-stage breast cancer.[4–7] The following two methods are commonly utilized for detecting SLNs: the radioimmunoassay (RI) method, which involves application of radioactive colloids, and injection of blue dye.[8,9] Both methods have their advantages and

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disadvantages.[10–12] The RI method has the advantage of a high SLN identification rate, while disadvantages include the requirement of a radioactive facility, exposure to radiation, and high cost. In contrast, the blue dye method has the advantages of a high prevalence rate, no radiation exposure, and low cost; however, SLN identification rates are lower with this method compared with the RI method.[13] Furthermore, the success of the blue dye method is dependent on the technician's skill and experience.[14]

Indocyanine green (ICG) is a dye on which laser-emitting diodes are centered at 760 nm to collect fluorescence at 830 nm. The fluorescing property of the ICG reagent was first applied to the dye method, followed by the fluorescence method.[15] The ICG fluorescence method requires a photodynamic eye (PDE) camera. It lacks the stringent safety controls of the RI method. Therefore, the fluorescence method is not limited to use in high-volume centers. Lymph flow can be confirmed as a real-time image from outside the body using the ICG fluorescence method; therefore, this method is well suited for performing intraoperative SLN biopsy.

Because the ICG fluorescence method requires little skill and the necessary reagents and apparatus are inexpensive in comparison with the RI method, use of the former method at the physician's discretion has been increasing. Recent clinical results obtained after introduction of the ICG fluorescence method have indicated higher SLN identification rates than those observed with the blue dye method.[16–19] However, operational procedures and experience of the personnel vary among institutions. In addition, no statistical analysis has clearly demonstrated the superiority of the ICG fluorescence method over the blue dye method. The present multicenter, cooperative, prospective analysis using a standardized procedure was performed to demonstrate the efficacy of the ICG fluorescence method in comparison with that of the blue dye method.

METHODS

Patients

Eligible patients were 20–75 years old at registration and diagnosed with T1–3 primary breast cancer without clinical lymph node metastasis (N0). Six participating centers in Japan have been governmentally authorized to perform SLN biopsy. SLN biopsies were performed by ten well-trained physicians according to a standard written procedure. This study was performed in accordance with the Declaration of Helsinki, and all patients provided written informed consent. The study protocol was approved by the local ethics committees at all participating trial sites. Patients in whom previous surgical biopsy or surgery involving the axillary regions had been performed, those in whom preoperative drug therapy (including hormone therapy and chemotherapy) had been administered, and those who had a history of allergy to ICG or indigo carmine dye were excluded from the study.

Surgical Procedure

All surgeons performed SLN biopsy following the standard procedure. In this study, SLNs were categorized as follows: axillary lymph nodes, blue-stained (true SLN); axillary lymph nodes, ICG fluorescence-positive detected by PDE (true SLN); and palpably suspicious, surgically removed lymph nodes in which neither ICG fluorescence nor blue dye was found (para-SLN). The surgeon's goal during the procedure was to remove the blue-stained and/or fluorescent lymph nodes (true SLNs) in the incised region. Palpated lymph nodes in the operative area were also removed as para-SLN.

The ICG fluorescence method has been previously reported.[17] In brief, 0.5–1 ml of 0.5 % ICG as a source of fluorescence and 2–4 ml of indigo carmine as a blue dye were injected in the subareolar area. Lymphatic flow was then traced with a PDE camera (a charge-coupled device; Hamamatsu Photonics Co., Hamamatsu, Japan). Real-time, image-guided surgery was used to identify the fluorescence signals of the SLNs after meticulous dissection. The excised lymph nodes were examined separately according to the order of removal and classified according to detection by ICG fluorescence and/or blue dye.

Study Objectives

The primary endpoint in this study was to determine the number of lymph nodes identified by each method. In each patient, all extracted lymph nodes were classified into four categories on the basis of the two detection methods as follows: SLNs identified by both fluorescence and blue dye (flu^+/dye^+), those identified by fluorescence only (flu^+/dye^-), those identified by dye only (flu^-/dye^+), and those in which neither fluorescence nor dye was observed (para-SLNs; flu^-/dye^-). Secondary endpoints included the SLN identification rate, SLN metastasis rate, and metastasis rate according to the order of SLN detection.

Statistical Methods

The number of lymph nodes identified using the ICG fluorescence method and that using the blue dye method were compared. Differences were calculated by subtracting the number of flu^-/dye^+ SLNs from the number of flu^+/dye^- SLNs for each patient. The sign test was used to test the null hypothesis that the number of identified lymph

nodes was equal. The identification rate via fluorescence or blue dye was defined by the proportion of patients with SLNs identified with either method. Exact 95 % confidence intervals were obtained on the basis of binomial distribution. Subgroup analyses of the SLN identification rate were conducted according to age and body mass index (BMI) using Fisher's exact test. Metastasis rates were separately obtained for flu^+/dye^+ SLNs, flu^+/dye^- SLNs, flu^-/dye^+ SLNs, and para-SLNs (flu^-/dye^-). They were also separately obtained for ordered flu^+ SLNs. The prevalence of adverse events was assessed. In order to use the sign test (alpha, 0.05; power, 0.90), results from 100 patients were required to detect whether the number of flu^+/dye^- SLNs was greater than that of flu^-/dye^+ SLNs in 66 % or more of eligible patients.

RESULTS

From February to October 2010, SLN biopsy was performed in 100 patients (mean age, 60 years; range, 29–75 years) with early-stage breast cancer. One patient was excluded in whom hormone therapy was administered before biopsy. Thus, 99 patients were eligible for further assessment. Of these, ductal carcinoma in situ was diagnosed in 7 %, while invasive ductal carcinoma was diagnosed in 93 %. Patient and tumor characteristics are summarized in Table 1.

Overall, the ICG fluorescence method identified an average of 3.4 SLNs in 98 of 99 patients (detection rate, 99 %). The median difference between the number of lymph nodes identified by the fluorescence and blue dye methods was one (range, 0–6 nodes), and the number of SLNs identified by the former method was significantly higher than that identified by the latter method ($p < 0.001$). Therefore, the SLN detection rate using the ICG fluorescence method was significantly higher than that by the dye method (99 vs. 78 %, $p < 0.001$; Table 2). Furthermore, SLN identification by fluorescence was independent of age and BMI (Table 3).

Table 4 summarizes the data obtained from the 99 patients ($n = 340$ SLN specimens) by the detection method. Of these patients, positive SLN identification was achieved by both methods in 78 % (77 of 99, flu^+/dye^+), ICG fluorescence alone detected SLNs in 69 % (68 of 99, flu^+/dye^-), and para-SLNs were identified in 35 % (35 of 99, flu^-/dye^-). In these 35 patients, true SLNs were identified using the ICG fluorescence and/or blue dye methods; in no patient were para-SLNs found alone. No SLNs were classified as flu^-/dye^+. Of the 340 specimens, true SLNs categorized as flu^+/dye^+ and flu^+/dye^- accounted for 36 % (121 of 340) and 47 % (160 of 340), respectively. Though para-SLNs (flu^-/dye^-) were identified in 17 % (59 of 340)

TABLE 1 Patients and tumor characteristics ($n = 99$)

Characteristic	Value
Age, years, mean (range)	60 (29–75)
Pathology	
Invasive ductal carcinoma	92 (93 %)
Noninvasive ductal carcinoma	7 (7 %)
Clinical tumor size	
Tis	4 (4 %)
T1a	4 (4 %)
T1b	15 (15 %)
T1c	39 (39 %)
T2	34 (34 %)
T3	1 (1 %)
Tx	2 (2 %)
Grade	
1	36 (36 %)
2	37 (37 %)
3	23 (23 %)
Unknown	3 (3 %)
Estrogen receptor	
Negative	17 (17 %)
Positive	81 (82 %)
Unknown	1 (1 %)
Progesterone receptor	
Negative	24 (24 %)
Positive	73 (74 %)
Unknown	2 (2 %)
HER2	
Negative	37 (37 %)
1+	32 (32 %)
2+	15 (15 %)
3+	12 (12 %)
Unknown	3 (3 %)
BMI (kg/m^2)	
<18.5	11 (11 %)
≥18.5, <22	39 (39 %)
≥22, <25	32 (32 %)
≥25 <30	13 (13 %)
≥30	4 (4 %)

BMI body mass index

TABLE 2 Comparison of sentinel lymph node detection between the ICG fluorescence method and the dye method

Characteristic	Result	*p*
Difference in number of lymph nodes identified		
Difference (ICG fluorescence–blue dye)	1.0 (range, 0–6)	<0.001
Detection rate		
ICG fluorescence	99 % (98/99)	<0.001
Dye	78 % (77/99)	

ICG indocyanine green

TABLE 3 SLN detection rate according to age and BMI using the ICG fluorescence method and the dye method

Characteristic	ICG (%)	p^a	Dye (%)	p^a
Age (years)				
<50	100 (30/30)	1.00	87 (26/30)	0.03
≥50, <60	100 (19/19)		95 (18/19)	
≥60, <70	97 (34/35)		69 (24/35)	
≥70	100 (15/15)		60 (9/15)	
BMI (kg/m^2)				
<18.5	100 (11/11)	0.61	100 (11/11)	0.20
≥18.5, <22	100 (39/39)		79 (31/39)	
≥22, <25	97 (31/32)		75 (24/32)	
≥25, <30	100 (13/13)		62 (8/13)	
≥30	100 (4/4)		75 (3/4)	

SLN sentinel lymph node, *BMI* body mass index, *ICG* indocyanine green

[a] Fisher's exact test

TABLE 4 Classification of SLN in terms of fluorescence and dye

Characteristic	Patients, % (n = 99)	SLNs identified, % (n = 340)
Flu$^+$/dye$^+$	78 (77/99)	36 (121/340)
Flu$^+$/dye$^-$	69 (68/99)	47 (160/340)
Flu$^-$/dye$^+$	0 (0/99)	0 (0/340)
Flu$^-$/dye$^-$	35 (35/99)	17 (59/340)

SLN sentinel lymph node, *flu* fluorescence

of these specimens, 100 % of the lymph nodes were ICG fluorescence-positive if para-SLNs were excluded.

Table 5 summarizes SLN and non-SLN involvement in order of SLN removal. The first SLN was defined as the proximal lymph node draining lymphatic flow from the tumor. Involvement of the first SLN was exhibited in all 20 % (20 of 99) of the patients with positive lymph nodes. Of these 20, the first isolated node was the only positive node in 12 (60 %; 9 flu$^+$/dye$^+$, 3 flu$^+$/dye$^-$). Metastases in the second or further SLNs were identified in eight patients, all of whom had a positive first SLN. In 16 of the 20 SLN-positive patients, complete ALND was performed. No axillary lymph node involvement was detected in all eight patients with the first SLN as the only positive lymph node, whereas non-SLN metastases were not detected in 4 of 8 (50 %) patients with positive second or further lymph nodes in addition to the first positive SLN. No skin necrosis or tattoo relevant to subcutaneous ICG injection was observed. No shock or other adverse reactions due to hypersensitivity were noted.

TABLE 5 SLN and non-SLN involvement in terms of the order of SLN removal

Characteristic	Patients, %
Patients with positive SLNs	20 (20/99)
Positive SLN identified by:	
ICG	100 (20/20)
Dye	70 (14/20)
First SLN positive alone	60 (12/20)
Completion of ALND	67 (8/12)
Non-SLN negative	100 (8/8)
Non-SLN positive	0 (0/8)
First SLN and second or further positive	40 (8/20)
Completion of ALND	100 (8/8)
Non-SLN negative	50 (4/8)
Non-SLN positive	50 (4/8)

SLN sentinel lymph node, *ICG* indocyanine green

DISCUSSION

This is the first prospective study to evaluate the efficacy of SLN detection using the ICG fluorescence method. The detection rate using this method was significantly higher than that using the indigo carmine or blue dye method in patients with early breast cancer. This high detection rate may be a consequence of the greater optical sensitivity of ICG compared to the color perception of the blue dye. In previous studies, the use of ICG without fluorescence imaging did not improve the SLN detection rate (73.8 %), whereas the ICG fluorescence method used in our study achieved a detection rate of 99 %, which was comparable to that reported in previous studies.[15–20]

The ICG fluorescence method uses an integrated dye coupled with an infrared camera equipped with a 765-nm wavelength emitter. Emitted near-infrared radiation activates ICG molecules and fluorescence emissions at a wavelength of 830 nm. This makes lymphatic flow and drainage of SLNs visible as fluorescence signals. Fluorescence imaging assists the surgeon in navigating the axillary basin along the subcutaneous vessels and enables orderly and sequential SLN dissection.

Obesity is associated with the development and recurrence of breast cancer.[21,22] Obesity may inhibit accurate identification of SLNs.[23,24] As the emitted fluorescence is more attenuated through fat droplets, fluorescence may decrease in proportion to increased body mass. In preliminary studies, detection of fluorescence signals deeper than 1 cm from the skin level was difficult. Abe et al.[25] reported a significant correlation between BMI and the time and depth required to reach SLNs in the axilla. In the current study, a stable SLN detection rate was observed using ICG fluorescence regardless of BMI, whereas the

detection rate using indigo carmine dye tended to decrease as BMI increased (NS). Thus, detection of SLN by ICG fluorescence is stable regardless of BMI, which may or may not reduce operative time compared to blue dye alone. The median BMI in patients included in the present study was 21.8 kg/m^2 (range, 17.6–32.4 kg/m^2). Only 4 % of patients had a BMI > 30 kg/m^2. As obesity is more prevalent in Caucasians than in Asians, the relationship between BMI and accuracy of SLN detection using ICG fluorescence requires further investigation in the former population. Skin compression techniques have been recently developed to ameliorate the attenuation of fluorescence signals. Skin compression is a simple procedure to overcome the weaknesses of the fluorescence technique in obese patients.[26]

Because lymph flow alters with age, older age may be significantly associated with false-negative SLN biopsy results. Cox et al.[23] reported that RI counts in SLNs were inversely correlated with age. In this study, the SLN detection rate using indigo carmine dye also decreased significantly with age, while the ICG fluorescence method achieved a stable and high detection rate, even in older patients.

In this study, fluorescence-positive and dye-positive (i.e., double positive) SLNs were detected in most patients (78 %). Using the ICG fluorescence method, the first SLN can be identified as the lymph node proximal to the tumor on the basis of drainage patterns and lymphatic flow. By contrast, with the RI method, identifying SLNs in order is difficult because they are detected as hot spots regardless of the anatomical lymphatic flow. In this study, all patients with SLN involvement had positive first SLNs. This means that the first SLN detected by ICG fluorescence imaging represents the exact axillary status.

The number of SLNs resected using fluorescence imaging tends to be higher than that resected using the RI method. In this study, the median number of resected SLNs was 3.4, which was greater than that (1.7–2) reported in studies using the RI method.[5–7] This disadvantage was because of the higher optical sensitivity of fluorescence imaging and the low molecular weight of ICG, which can spread further within the lymphatic basin than blue dye. However, a positive SLN is usually identified within the first 4 resected SLNs.[27] Removal of ≤4 nodes is acceptable for optimal accuracy of SLN biopsy.[28,29]

The avoidance of routine application of ALND in patients with positive SLNs is currently under debate in terms of breast cancer outcome. If the tumor burden on SLNs is low, locoregional recurrence can be controlled by irradiation, adjuvant chemotherapy, and hormonal therapy. The Austrian Breast and Colorectal Cancer Study Group (ABCSG Z0011) study reported outcomes of ≤2 SLN-positive patients in both ALND and non-ALND cohorts.[30] If axillary clearance was omitted in patients who met the ABCSG Z0011 criteria, three or more SLNs could be resected, which is compatible with the ICG fluorescence method.

In this study, ALND procedures were unnecessarily performed in 75 % (12 of 16) of the patients with positive SLNs, whereas residual axillary disease was found in the remaining 25 % (4 of 16). Of the eight patients with a positive lymph node isolated as the first SLN, none had residual axillary disease. These results suggested that SLN biopsy can safely replace axillary clearance for surgeons otherwise willing to perform further axillary treatment. However, a direct comparison between the ICG fluorescence method and the RI method is required for ensuring the efficacy of the former method. A small study ($n = 30$) has already reported a high SLN detection rate using the ICG fluorescence method. Use of this method decreased the false-negative rate associated with the RI method when both methods were combined.[31] A study using three tracer agents (RI, blue dye, and ICG) recently confirmed the combination of ICG and blue dye method had the highest nodal sensitivity, which avoids the need for radioisotopes.[32] A large-scale prospective study is currently ongoing to test the concordance between these methods. This study may lead to a new proposal for the optimal method of SLN detection and subsequent axillary management in patients with early-stage breast cancer.

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REFERENCES

1. Schrenk P, Rieger R, Shamiyeh A, et al. Morbidity following sentinel lymph node biopsy versus axillary lymph node dissection for patients with breast carcinoma. *Cancer.* 2000;88:608–14.
2. Giuliano AE, Haigh PI, Brennan MB, et al. Prospective observational study of sentinel lymphadenectomy without further axillary dissection in patients with sentinel node-negative breast cancer. *J Clin Oncol.* 2000;18:2553–9.
3. Purushotham AD, Upponi S, Klevesath MB, et al. Morbidity after sentinel lymph node biopsy in primary breast cancer: results from a randomized controlled trial. *J Clin Oncol.* 2005;23:4312–21.
4. Morton DL, Wen DR, Wong JH, et al. Technical details of intraoperative lymphatic mapping for early stage melanoma. *Arch Surg.* 1992;127:392–9.
5. Veronesi U, Paganelli G, Viale G, et al. A randomized comparison of sentinel-node biopsy with routine axillary dissection in breast cancer. *N Engl J Med.* 2003;349:546–53.
6. Krag DN, Anderson SJ, Julian TB, et al. Sentinel-lymph-node resection compared with conventional axillary-lymph-node dissection in clinically node-negative patients with breast cancer: overall survival findings from the NSABP B-32 randomized phase 3 trial. *Lancet Oncol.* 2010;11:927–33.
7. Mansel RE, Fallowfield L, Kissin M, et al. Randomized multicenter trial of sentinel node biopsy versus standard axillary treatment in operable breast cancer: the ALMANAC trial. *J Natl Cancer Inst.* 2006;98:599–609.

8. Krag DN, Weaver DL, Alex JC, et al. Surgical resection and radio-localization of the sentinel node in breast cancer using a gamma probe. *Surg Oncol.* 1993;2:335–40.
9. Giuliano AE, Kirgan DM, Guenther JM, et al. Lymphatic mapping and sentinel lymphadenectomy for breast cancer. *Ann Surg.* 1994;220:391–401.
10. McMasters KM, Tuttle TM, Carlson DJ, et al. Sentinel lymph node biopsy for breast cancer: a suitable alternative to routine axillary dissection in multi-institutional practice when optimal technique in used. *J Clin Oncol.* 2000;18:2560–6.
11. Cody HS 3rd, Fey J, Akhurst T, et al. Complementarity of blue dye and isotope in sentinel node localization for breast cancer: univariate and multivariate analysis of 966 procedures. *Ann Surg Oncol.* 2001;18:2560–6.
12. Tafra L, Lannin DR, Swanson MS, et al. Multicenter trial of sentinel node biopsy for breast cancer using both technetium sulfur colloid and isosulfan blue dye. *Ann Surg.* 2001;233:51–9.
13. Kin T, Guiliano AE, Lyman GH. Lymphatic mapping and sentinel lymph node biopsy in early-stage breast carcinoma. *Cancer.* 2006;106:4–16.
14. Morrow M, Rademaker AW, Bethke KP, et al. Learning sentinel node biopsy: results of a prospective randomized trial of two techniques. *Surgery.* 1999;126:714–20.
15. Kitai T, Inomoto T, Miwa M, Shikayama T. Fluorescence navigation with indocyanine green for detecting sentinel lymph nodes in breast cancer. *Breast Cancer.* 2005;12:211–5.
16. Tagaya N, Yamazaki R, Nakagawa A, et al. Intraoperative identification of sentinel lymph nodes by near-infrared fluorescence imaging inpatients with breast cancer. *Am J Surg.* 2008; 195:850–3.
17. Sugie T, Kassim KA, Takeuchi M, et al. A novel method for sentinel lymph node biopsy by indocyanine green fluorescence technique in breast cancer. *Cancers.* 2010;2:713–20.
18. Hojo T, Nagao T, Kikuyama M, et al. Evaluation of sentinel node biopsy by combined fluorescent and dye method and lymph flow for breast cancer. *Breast.* 2010;19:210–3.
19. Hishch C, Murawa D, Mohr Z, et al. ICG fluorescence-guided sentinel node biopsy for axillary nodal staging in breast cancer. *Breast Cancer Res Treat.* 2010;121:373–8.
20. Motomura K, Inaji H, Komoike Y, et al. Sentinel node biopsy guided by indocyanine green dye in breast cancer patients. *Jpn J Clin Oncol.* 1999;29:604–7.
21. Anderson GL, Neuhouser ML. Obesity and the risk for premenopausal and postmenopausal breast cancer. *Cancer Prev Res (Phila).* 2012;5:515–21.
22. Healy LA, Ryan AM, Rowley S, et al. Obesity increases the risk of postmenopausal breast cancer and is associated with more advanced stage at presentation but no impact on survival. *Breast J.* 2010;16:95–7.
23. Cox CE, Dupont E, Whitehead GF, et al. Age and body mass index may increase the chance of failure in sentinel lymph node biopsy for women with breast cancer. *Breast J.* 2002;8:88–91.
24. Hughes M, Goffman TG, Perry RR, et al. Obesity and lymphatic mapping with sentinel lymph node biopsy in breast cancer. *Am J Surg.* 2004;187:52–7.
25. Abe H, Umeda T, Mori M, et al. Indocyanine green fluorescence imaging system for sentinel lymph node biopsy in early breast cancer patients. *J Clin Oncol.* 2010;28(Suppl. 15):6492.
26. Kitai T, Kawashima M. Transcutaneous detection and direct approach to the sentinel node using axillary compression technique in ICG fluorescence-navigated sentinel node biopsy for breast cancer. *Breast Cancer.* 2012;19:343–8.
27. Zakaria S, Degnim AC, Kleer CG, et al. Sentinel lymph node biopsy for breast cancer: how many nodes are enough? *J Surg Oncol.* 2007;96:554–9.
28. Toi M, Winer EP, Inamoto T, et al. Identifying gaps in the locoregional management of early breast cancer: highlights from the Kyoto Consensus Conference. *Ann Surg Oncol.* 2011;18: 2885–92.
29. Ban EJ, Lee JS, Koo JS, et al. How many sentinel lymph nodes are enough for accurate axillary staging in t1–2 breast cancer? *J Breast Cancer.* 2011;14:296–300.
30. Giuliano AE, Hunt KK, Ballman KV, et al. Axillary dissection vs no axillary dissection in women with invasive breast cancer and sentinel node metastasis: a randomized clinical trial. *JAMA.* 2011;305:569–75.
31. Murawa D, Hirche C, Dresel S, et al. Sentinel lymph node biopsy in breast cancer guided by indocyanine green fluorescence. *Br J Surg.* 2009;96:1289–94.
32. Wishart GC, Loh SW, Jones L, et al. A feasibility study (ICG-10) of indocyanine green (ICG) fluorescence mapping for sentinel lymph node detection in early breast cancer. *Eur J Surg Oncol.* 2012;38:651–6.

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Original Article

p53 Expression in Pretreatment Specimen Predicts Response to Neoadjuvant Chemotherapy Including Anthracycline and Taxane in Patients with Primary Breast Cancer

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While clinical and pathologic responses are important prognostic parameters, biological markers from core needle biopsy (CNB) are needed to predict neoadjuvant chemotherapy (NAC) response, to individualize treatment, and to achieve maximal efficacy. We retrospectively evaluated the cases of 183 patients with primary breast cancer who underwent surgery after NAC (anthracycline and taxane) at the National Cancer Center Hospital (NCCH). We analyzed EGFR, HER2, and p53 expression and common clinicopathological features from the CNB and surgical specimens of these patients. These biological markers were compared between sensitive patients (pathological complete response; pCR) and insensitive patients (clinical no change; cNC and clinical progressinve disease; cPD). In a comparison between the 9 (5%) sensitive patients and 30 (16%) insensitive patients, overexpression of p53 but not overexpression of either HER2 or EGFR was associated with a good response to NAC. p53 ($p = 0.045$) and histological grade 3 ($p = 0.011$) were important and significant predictors of the response to NAC. The correspondence rates for histological type, histological grade 3, ER, PgR, HER2, p53, and EGFR in insensitive patients between CNB and surgical specimens were 70%, 73%, 67%, 70%, 80%, 93%, and 73%. The pathologic response was significantly associated with p53 expression and histological grade 3. The correspondence rate of p53 expression between CNB and surgical specimens was higher than that of other factors. We conclude that the level of p53 expression in the CNB was an effective and reliable predictor of treatment response to NAC.

Key words: breast cancer, neoadjuvant chemotherapy, predictors

Neoadjuvant chemotherapy (NAC) is the standard therapy for patients with advanced local breast cancer and is used increasingly for operable disease. Clinical and pathologic responses are important prognostic parameters, but cannot be accurately predicted. Unfortunately, approximately 20% of breast cancer patients do not benefit from NAC (*i.e.*, they continue to show stable or progressive disease). One of the aims of NAC is to confirm the sensitivity of tumors to chemotherapy. Using NAC, we can directly determine the sensitivity to chemotherapy based on whether or not the primary tumor is diminished, whereas we

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cannot confirm the efficacy by adjuvant chemotherapy itself. However, non-sensitive patients have to endure relatively needless therapy for about 6 months, so it is very important to make the pre-diagnosis of sensitivity to chemotherapy if possible. Several biological markers that might predict response are under investigation [1-9]. Estrogen receptor, progesterone receptor, and HER2 are very useful markers for the selection of anticancer drugs and prediction of prognosis, but are not useful for predicting the response to chemotherapeutic agents such as anthracycline and taxane. Therefore, other biological markers from pre-treatment core needle biopsy are needed to predict the response to NAC, to individualize treatment, and to achieve maximal efficacy.

In this study, we investigated biological markers from pre-treatment core needle biopsies of highly sensitive tumors and non-sensitive tumors and identified additional prognostic markers that might predict the response to NAC and aid in the selection of treatment strategy.

Materials and Methods

All patients with operable breast cancer who were treated between May 1998 and July 2006 at the National Cancer Center Hospital with anthracycline and/or taxane as NAC were included in this retrospective study. NAC was indicated for clinical stage II breast cancer patients with tumors larger than 3 cm and stage III breast cancer patients. Core needle biopsy was performed before NAC to allow pathological diagnosis. Doxorubicin (DOX, 50 mg/m^2) and docetaxel (DTX, 60 mg/m^2) were administered for four 3-week cycles before surgery. Additional adjuvant treatment with DOX/DTX was given if patients achieved complete or partial remission after NAC. Otherwise, patients were treated with four cycles of iv cyclophosphamide, methotrexate, and 5FU. Trastuzumab was not administered to the patients with HER2-overexpressing tumors. Tamoxifen (20 mg/day) or anastrozole (10 mg/day) was administered for 5 years after surgery if either the pretreatment biopsy specimen or the surgical specimen post-chemotherapy was positive for estrogen-receptor or progesterone receptor.

Pretreatment diagnosis was established by our pathologists using samples from core needle biopsy or surgical resection. Overexpression of hormone receptors, p53, HER2 and EGFR was examined by immunohistology. Surgical specimens were sectioned at about 7-10 mm and classified for pathological response. Pathological features were described and invasive ductal carcinomas were classified into 3 subtypes (papillotubular, solid tubular, and scirrhous) according to the General and Pathological Recording of Breast Cancer guidelines established by the Japanese Breast Cancer Society [10]. The criteria for histological grading of IDC were based on a modification of those recommended by the WHO [11, 12]. The response criteria used in this study include Fisher's system [13], complete pCR denotes no histological evidence of tumor cells, pCR with DCIS denotes no histological evidence of invasive tumor cells (specimens with only noninvasive cells included), and pINV denotes the presence of invasive tumor cells. Overexpression of ER (1D5, Dako Cytomation, Baltimore, MD, USA), PgR (1A6, Novocastra), HER2 (Herceptest, Dako), p53 (DO7, Dako), and EGFR (2-18C9, Dako) were examined by immunohistology using the noted antibodies. The criterion for ER, PgR, and p53 was staining of more than 10% of cancer cell nuclei, regardless of intensity. HER2 and EGFR grading is as follows: 0: negative, 1+: slightly positive in more than 10% of cancer cells, 2+: moderately positive in more than 10% of cancer cells, 3+: markedly positive in more than 10% of cancer cells. 2+ and 3+ were considered positive for HER2 and EGFR.

Clinical response to NAC was decided from the 2 greatest perpendicular diameters (before each chemotherapy treatment and before surgery) of tumors in the breast and axillary lymph nodes. Absence of clinical evidence of palpable tumors in the breast and axillary lymph nodes was defined as a clinical complete response (cCR). Reduction in total tumor size of 30% or greater was graded as clinical partial response (cPR). An increase in total tumor size of more than 20% or appearance of new suspicious ipsilateral axillary adenopathy was considered progressive disease (cPD). Tumors that did not meet the criteria for objective response or progression were classified as stable disease (cSD). In this study, we analyzed biological markers from core needle biopsies before NAC in complete pCR cases and non-sensitive tumors (clinical SD and PD), and demonstrated bio-

logical predictors of pathological response to PST.

Statistical analysis was carried out using JMP version 6.0 (SAS Institute Inc., Cary, NC, USA). Associations between ordinal variables were assessed using χ^2 analyses or the Fisher exact test for two-by-two variables. The statistical significance (P) was taken as a measure of the strength of evidence against the null hypothesis, and $p \leq .05$ was considered statistically significant.

Results

One hundred and eighty-three patients with operable breast cancer were treated with NAC at National Cancer Center Hospital between May 1998 and October 2001. Table 1 lists the patient and tumor characteristics. The median age was 50 years (range: 29–70). At diagnosis, 41 (22%) patients were in stage IIA, 63 (34%) were in stage IIB, 37 (20%) were in stage IIIA, and 42 (23%) were in stage IIIB. Breast conserving surgery was performed for 55 (30%) patients after NAC. The overall clinical response rate to NAC was 83% (cCR+cPR) and the pCR rate was 13%. 30 (17%) patients were insensitive to NAC (cSD or cPD). Among the responsive patients, 9 (5%) exhibited complete pCR (pathologically no tumor in the breast) and 14 (8%) exhibited pCR with DCIS.

Immunohistological characteristics from core needle biopsy before NAC are listed in Table 2. There were 62 (34%) cases of solid tubular primary tumor, 65 (36%) scirrhous, 34 (19%) papillotubular, 9 (5%) ILC, and 3 (2%) mucinous carcinomas. 88 (48%) cases were histological grade 3. 66 (36%) were ER positive and 72 (39%) were PgR positive. 73 (40%) were HER-2 positive (2+ and 3+ in immunohistological examination).

We evaluated age, histological type, histological grade, ER, PgR, HER2, EGFR, and p53 as predictive factors for response to NAC by comparing 9 (5%) sensitive (complete pCR) and 30 (17%) insensitive (cSD and cPD) tumors (Table 3). In univariate analysis, histological grade 3 ($p = 0.011$) and p53 ($p = 0.045$) were significant predictors of complete pCR. However, EGFR and HER2 were not predic-

Table 1 Patient and tumor characteristics

Parameter	No. of patients (%)
Total	183
Age (median)	50 (29–70)
Clinical stage	
Stage IIA	41 (22%)
Stage IIB	63 (34%)
Stage IIIA	37 (20%)
Stage IIIB	42 (23%)
Operation	
Bt + Ax	128 (70%)
Bp + Ax	55 (30%)
Clinical response	
cCR	32 (17%)
cPR	121 (66%)
cNC	29 (16%)
cPD	1 (1%)
Pathological response	
complete pCR	9 (5%)
pCR with DCIS	14 (8%)
pINV	160 (87%)

Bt, total mastectomy; Bp, partial mastectomy; Ax, axillary lymph node dissection.

Table 2 Immunohistological characteristics of CNB before PST

Parameter	No. of patients (%)
Histological type	
IDC	161 (88)
Solid tubular	62 (34)
Scirrhous	65 (36)
Papillotubular	34 (19)
ILC	9 (5)
mucinous	3 (2)
others	10 (5)
Histological grade	
3	88 (48)
2	88 (48)
1	7 (4)
ER	
positive	66 (36)
negative	117 (64)
PgR	
positive	72 (39)
negative	111 (61)
HER2	
positive (2 + and 3 +)	73 (40)

tors.

We analyzed the immunohistological features of CNB specimens. The correspondence rates of these features in insensitive patients between CNB and surgical specimens are shown in Table 4. The correspondence rates for histological type, histological grade 3, ER, PgR, HER2, p53, and EGFR were 70%, 73%, 67%, 70%, 80%, 93%, and 73%. The correspondence rate of EGFR was not low; however, in almost all patients with a discrepancy between CNB and surgical specimens, EGFR overexpression changed from negative to positive.

Discussion

The identification of predictive factors for NAC is very important for order made cancer treatment. The development of new medicines has diversified chemotherapeutic regimens, and the selection of treatment strategy according to individual cancer characteristics has become more difficult. To aid in selection, translational research has begun to demonstrate important correlations between prognostic factors and sensitivity to chemotherapy.

Table 4 Correspondence rates of biological markers in insensitive patients between CNB and surgical specimens

Parameter	%
Histological type	70
Histological grade 3	73
ER	67
PgR	70
HER2	80
p53	93
EGFR	73

In this study, we retrospectively evaluated response to NAC including anthracycline and taxane and a number of biomarkers. We found that pathologic response significantly associated with p53 expression and histological grade 3.

In our analysis, p53 could predict response of NAC. p53 accumulation was reported to be associated with a poor response to anthracycline in node-negative breast cancer patients [14], and may compromise the efficacy of anthracycline but not of taxane [15]. All patients in this study received both anthracycline and taxane, and p53 was an independent predictive factor of response to NAC similar to these reports. We cannot analyze the response of anthracycline and taxane respectively. However commonly we use both drugs in NAC. If the tumor has p53 mutation before NAC, we should check the response of anthracycline tightly and change to taxane when the response is wrong.

Previous studies reported poor prognosis for patients with HER2-overexpression. Several studies indicate that HER2 expression can predict sensitivity to anthracycline chemotherapy [16]; however, in this study, HER2 was not a predictor of pCR to NAC. HER2 negative patients rate were 22% of good responders and 33% of poor responders. In this study trastuzumab was not administered to patients with HER2 overexpression tumors. However, in these days, trastuzumab significantly improved the prognosis and the response to chemotherapy in these patients [17]. It was reported that the rate of pCR patients administered trastuzumab was significantly high. HER2 expression was not predictor of response to anthracycline and taxane in this study. We need to examine the relationship between HER2-overexpression and response to chemotherapy with trastuzumab.

Table 3 Univariate analysis of clinicopathological features between sensitive (pCR) and insensitive cases (cNC + cPD)

Parameter	Sensitive (n = 9) (%)	Non-sensitive (n = 30) (%)	*p*-value
Age < 50	3 (33)	19 (63)	N.S.
Histological type (so.)	6 (67)	12 (40)	N.S.
Histological grade 3	8 (89)	13 (43)	0.011
ER negative	8 (89)	17 (57)	N.S.
PgR negative	6 (67)	17 (57)	N.S.
HER2 positive	2 (22)	10 (33)	N.S.
p53 positive	5 (56)	6 (20)	0.045
EGFR positive	3 (33)	7 (23)	N.S.

so, solid tubular carcinoma

A previous study observed EGFR expression in 37-80% of basal-like tumors, as identified by DNA microarray, and reported poorer prognosis for this phenotype [18-20]. We hypothesized that EGFR expression might distinguish the basal-like phenotype and predict poorer response to NAC. However, in this study, EGFR was not an independent predictive factor of response to NAC. It was reported that EGFR is expressed in 7-36% of breast carcinomas with high grade conventional invasive ductal carcinoma (IDC) [21-24] and EGFR expression was seen in 272 (20%) of 1388 cases. In a univariate analysis, Tsutsui *et al.* showed a significantly poorer clinical outcome for patients with EGFR-positive tumors compared with those who were EGFR-negative, both for overall survival and disease-free survival [21]. The correspondence rate of EGFR overexpression between core needle biopsy and surgical specimens was higher than the correspondence rates of common predictive factors (ER, PgR, and HER2) between the 2 types of specimens. However, the rates of EGFR expression were relatively low in both sensitive (33%) and insensitive patients (23%). In addition, in cases in which EGFR expression did not correspond between CNB and surgical specimens, EGFR was always negative in CNB, but positive in the surgical specimen. Therefore, it is possible that core needle biopsy specimens are inadequate to evaluate EGFR overexpression, or that EGFR expression was stimulated by chemotherapy. Following NAC, highly malignant EGFR-positive tumor cells increased in number, while EGFR-negative cells decreased in number. In these specimens, other common predictive factors did not change pre- and post-NAC; therefore it is not certain that all of the CNB specimens were inadequate. Indeed, it may be that NAC changed the characteristics of some tumors.

We evaluated EGFR, HER2, p53 and other common markers in specimens from pretreatment core needle biopsies as predictors of response to NAC. p53 was a more significant predictor than ER and histological grade, factors that have been previously reported. These results may have been influenced by the uncertainty of core needle biopsy results and the heterogeneity of cancer cells in the tumors. The correspondence rates of these common markers between CNB and surgical specimens were relatively low. However, the correspondence rate of p53 was significantly high. This result indicates that p53 is a stable parameter and suitable for predicting the response to neoadjuvant chemotherapy and for pretreatment diagnosis from CNB specimens.

Pretreatment diagnosis from CNB specimens is necessary to decide the strategy for primary breast cancer treatment. Therefore, identifying prognostic factors is very important, and we need a greater sample size to establish a classification system to predict patient outcome.

References

1. Burcombe RJ, Makris A, Richman PI, Daley FM, Noble S, Pittam M, Wright D, Allen SA, Dove J and Wilson GD: Evaluation of ER, PgR, HER-2 and Ki-67 as predictors of response to neoadjuvant Anthracycline chemotherapy for operable breast cancer. Br J Cancer (2005) 92: 147-155.
2. Petit T, Wilt M, Velten M, Millon R, Rodier JF, Borel C, Mors R, Haegele P, Eber M and Ghnassia JP: Comparative value of tumour grade, hormonal receptors, Ki-67, HER-2 and topoisomerase II alpha status as predictive markers in breast cancer patients treated with neoadjuvant Anthracycline-based chemotherapy. Eur J Cancer (2004) 40: 205-211.
3. Amat S, Abrial C, Penault-Llorca F, Delva R, Bougnoux P, Leduc B, Mouret-Reynier MA, Mery-Mignard D, Bleuse JP, Dauplat J, Cure H and Chollet P: High prognostic significance of residual disease after neoadjuvant chemotherapy: a retrospective study in 710 patients with operable breast cancer. Breast Cancer Res Treat (2005) 94: 255-263.
4. Chollet P, Amat S, Cure H, de Latour M, Le Bouedec G, Mouret-Reynier MA, Ferriere JP, Achard JL, Dauplat J and Penault-Llorca F: Prognostic significance of a complete pathological response after induction chemotherapy in operable breast cancer. Br J Cancer (2002) 86: 1041-1046.
5. Vincent-Salomon A, Rousseau A, Jouve M, Beuzeboc P, Sigal-Zafrani B, Fréneaux P, Rosty C, Nos C, Campana F, Klijanienko J, Al Ghuzlan A and Sastre-Garau X: Breast Cancer Study Group. Proliferation markers predictive of the pathological response and disease outcome of patients with breast carcinomas treated by anthracycline-based preoperative chemotherapy. Eur J Cancer (2004) 40: 1502-1508.
6. Burcombe R, Wilson GD, Dowsett M, Khan I, Richman PI, Daley F, Detre S and Makris A: Evaluation of Ki-67 proliferation and apoptotic index before, during and after neoadjuvant chemotherapy for primary breast cancer. Breast Cancer Res (2006) 8: R31.
7. Prisack HB, Karreman C, Modlich O, Audretsch W, Danae M, Rezai M and Bojar H: Predictive biological markers for response of invasive breast cancer to anthracycline/cyclophosphamide-based primary (radio-)chemotherapy. Anticancer Res (2005) 25: 4615-4621.
8. Ogston KN, Miller ID, Schofield AC, Spyrantis A, Pavlidou E, Sarkar TK, Hutcheon AW, Payne S and Heys SD: Can patients' likelihood of benefiting from primary chemotherapy for breast cancer be predicted before commencement of treatment? Breast Cancer Res Treat (2004) 86: 181-189.
9. Tiezzi DG, Andrade JM, Ribeiro-Silva A, Zola FE, Marana HR and Tiezzi MG: HER-2, p53, p21 and hormonal receptors proteins

expression as predictive factors of response and prognosis in locally advanced breast cancer treated with neoadjuvant docetaxel plus epirubicin combination. BMC Cancer (2007) 7: 36.
10. Sakamoto G, Inaji H, Akiyama F, Haga S, Hiraoka M, Inai K, Iwase T, Kobayashi S, Sakamoto G, Sano M, Sato T, Sonoo H, Tsuchiya S and Watanabe T: Japanese Breast Cancer Society. Japanese breast cancer society. General rules for clinical and pathological recording of breast cancer. Breast Cancer (2005) 12: S12-14.
11. Histological Typing of Breast Tumours. International Histological Classification of Tumours. No. 2, World Health Organization Geneva (1981) pp18-22.
12. Tsuda H, Sakamaki C, Tsugane S, Fukutomi T and Hirohashi S: Prognostic significance of accumulation of gene and chromosome alterations and histological grade in node-negative breast carcinoma. Jpn J Clin Oncol (1998) 28: 5-11.
13. Fisher B, Bryant J and Wolmark N: Effect of preoperative chemotherapy on the outcome of women with operable breast cancer. J Clin Oncol (1998) 16: 2672-2685.
14. Clahsen PC, van de Velde CJ, Duval C, Pallud C, Mandard AM, Delobelle-Deroide A, van den Broek L, Sahmoud TM and van de Vijver MJ: p53 protein accumulation and response to adjuvant chemotherapy in premenopausal women with node-negative early breast cancer. J Clin Oncol (1998) 16: 470-479.
15. Di Leo A, Tanner M, Desmedt C, Paesmans M, Cardoso F, Durbecq V, Chan S, Perren T, Aapro M, Sotiriou C, Piccart MJ, Larsimont D and Isola J: TAX 303 translational study team. p-53 gene mutations as a predictive marker in a population of advanced breast cancer patients randomly treated with doxorubicin or docetaxel in the context of a phase III clinical trial. Ann Oncol (2007) 18: 997-1003.
16. Gennari A, Sormani MP, Pronzato P, Puntoni M, Colozza M, Pfeffer U and Bruzzi P: HER2 status and efficacy of adjuvant anthracyclines in early breast cancer: a pooled analysis of randomized trials. J Natl Cancer Inst (2008) 100: 14-20.
17. Buzdar AU, Ibrahim NK, Francis D, Booser DJ, Thomas ES, Theriault RL, Pusztai L, Green MC, Arun BK, Giordano SH, Cristofanilli M, Frye DK, Smith TL, Hunt KK, Singletary SE, Sahin AA, Ewer MS, Buchholz TA, Berry D and Hortobagyi GN: Significantly higher pathologic complete remission rate after neoadjuvant therapy with trastuzumab, paclitaxel, and epirubicin chemotherapy: results of a randomized trial in human epidermal growth factor receptor 2-positive operable breast cancer. J Clin Oncol (2005) 23: 3676-3685.
18. Fan C, Oh DS, Wessels L, Weigelt B, Nuyten DS, Nobel AB, van't Veer LJ and Perou CM: Concordance among gene-expression-based predictors for breast cancer. N Engl J Med (2006) 355: 560-569.
19. Rouzier R, Perou CM, Symmans WF, Ibrahim N, Cristofanilli M, Anderson K, Hess KR, Stec J, Ayers M, Wagner P, Morandi P, Fan C, Rabiul I, Ross JS, Hortobagyi GN and Pusztai L: Breast cancer molecular subtypes respond differently to preoperative chemotherapy. Clin Cancer Res (2005) 11: 5678-5685.
20. Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, Hernandez-Boussard T, Livasy C, Cowan D, Dressler L, Akslen LA, Ragaz J, Gown AM, Gilks CB, van de Rijn M and Perou CM: Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin Cancer Res (2004) 10: 5367-5374.
21. Tsutsui S, Ohno S, Murakami S, Hachitanda Y and Oda S: Prognostic value of epidermal growth factor receptor (EGFR) and its relationship to the estrogen receptor status in 1029 patients with breast cancer. Breast Cancer Res Treat (2002) 71: 67-75.
22. Walker RA and Dearing SJ: Expression of epidermal growth factor receptor mRNA and protein in primary breast carcinomas. Breast Cancer Res Treat (1999) 53: 167-176.
23. Shien T, Tashiro T, Omatsu M, Masuda T, Furuta K, Sato N, Akashi-Tanaka S, Uehara M, Iwamoto E, Kinoshita T, Fukutomi T, Tsuda H and Hasegawa T: Frequent overexpression of epidermal growth factor receptor (EGFR) in mammary high grade ductal carcinomas with myoepithelial differentiation. J Clin Pathol (2005) 58: 1299-1304.
24. Hoadley KA, Weigman VJ, Fan C, Sawyer LR, He X, Troester MA, Sartor CI, Rieger-House T, Bernard PS, Carey LA and Perou CM: EGFR associated expression profiles vary with breast tumor subtype. BMC Genomics (2007) 8: 258.

Original Article

Analysis of Ki-67 Expression With Neoadjuvant Anastrozole or Tamoxifen in Patients Receiving Goserelin for Premenopausal Breast Cancer

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BACKGROUND: The increasing costs associated with large-scale adjuvant trials mean that the prognostic value of biologic markers is increasingly important. The expression of nuclear antigen Ki-67, a marker of cell proliferation, has been correlated with treatment efficacy and is being investigated for its value as a predictive marker of therapeutic response. In the current study, the authors explored correlations between Ki-67 expression and tumor response, estrogen receptor (ER) status, progesterone receptor (PgR) status, and histopathologic response from the STAGE study (Study of Tamoxifen or Arimidex, combined with Goserelin acetate to compare Efficacy and safety). **METHODS:** In a phase 3, double-blind, randomized trial (National Clinical Trials identifier NCT00605267), premenopausal women with ER-positive, early stage breast cancer received either anastrozole plus goserelin or tamoxifen plus goserelin for 24 weeks before surgery. The Ki-67 index, hormone receptor (ER and PgR) status, and histopathologic responses were determined from histopathologic samples that were obtained from core-needle biopsies at baseline and at surgery. Tumor response was determined by using magnetic resonance imaging or computed tomography. **RESULTS:** In total, 197 patients were randomized to receive either anastrozole plus goserelin (n = 98) or tamoxifen plus goserelin (n = 99). The best overall tumor response was better for the anastrozole group compared with the tamoxifen group both among patients who had a baseline Ki-67 index ≥20% and among those who had a baseline Ki-67 index <20%. There was no apparent correlation between baseline ER status and the Ki-67 index in either group. Positive PgR status was reduced from baseline to week 24 in the anastrozole group. **CONCLUSIONS:** In premenopausal women with ER-positive breast cancer, anastrozole produced a greater best overall tumor response compared with tamoxifen regardless of the baseline Ki-67 index. *Cancer* **2013;119:704-13.** © *2012 American Cancer Society.*

KEYWORDS: anastrozole, aromatase inhibitor, biomarker, neoadjuvant, Ki-67, premenopausal breast cancer.

INTRODUCTION

In addition to ablative surgery, radiotherapy, and cytotoxic chemotherapy, an additional standard treatment option for premenopausal women with estrogen receptor (ER)-positive breast cancer is the ER antagonist tamoxifen, either alone or in combination with ovarian function suppression.[1] Temporary and potentially reversible ovarian suppression can be achieved by treatment with a luteinizing hormone-releasing hormone analog, such as goserelin. Goserelin in combination with tamoxifen has demonstrated improved progression-free survival and disease-free survival compared with goserelin alone in premenopausal women with hormone receptor-positive (ER-positive and/or progesterone receptor [PgR]-positive) breast cancer in the advanced[2] and adjuvant[3] settings.

Nonsteroidal aromatase inhibitors (AIs), including anastrozole and letrozole, and the irreversible steroidal aromatase inactivator exemestane have demonstrated improved efficacy compared with tamoxifen in the advanced[4-7] and adjuvant[8-

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[12] treatment settings. Therefore, AIs in combination with ovarian suppression have been evaluated for the treatment of premenopausal women with ER-positive breast cancer.[13,14]

Neoadjuvant treatment for breast cancer provides an opportunity for downstaging of large tumors to allow patients to undergo breast-conserving surgery rather than mastectomy. Chemotherapy can offer an effective neoadjuvant treatment; however, increasing evidence suggests that ER-positive tumors are less sensitive to chemotherapy.[15] It has been demonstrated that neoadjuvant endocrine therapy has efficacy in the treatment of ER-positive disease among postmenopausal women, resulting in similar objective response rates and rates of breast-conserving surgery for AIs compared with more cytotoxic chemotherapy.[16] Therefore, the role of neoadjuvant endocrine therapy in premenopausal women is also of interest.

With the increasing costs associated with large-scale adjuvant trials, both the prognostic value of biologic markers and the long-term predictive value of short-term trials are increasingly important. The expression of nuclear antigen Ki-67, a marker of cell proliferation, reportedly has been correlated with treatment efficacy and is being investigated for its value as a predictive marker of therapeutic response.[17] In a cross-trial comparison, an increased reduction in Ki-67 expression after neoadjuvant treatment with anastrozole compared with tamoxifen was observed consistently; and increased progression-free survival has been reported for anastrozole versus tamoxifen in the adjuvant Arimidex, Tamoxifen, Alone or in Combination (ATAC) trial.[8,18,19]

The STAGE study (Study of Tamoxifen or Arimidex Combined With Goserelin Acetate to Compare Efficacy and Safety) was the first randomized trial to compare anastrozole plus goserelin versus tamoxifen plus goserelin in the neoadjuvant setting (24 weeks of therapy) in premenopausal women with ER-positive and human epidermal growth factor receptor 2 (HER2)-negative, operable breast cancer. The patients who received anastrozole plus goserelin in that trial had a superior best overall tumor response compared with the patients who received tamoxifen plus goserelin, as measured on magnetic resonance imaging (MRI) or computed tomography (CT) studies (anastrozole plus goserelin, 64.3%; tamoxifen plus goserelin, 37.4%; estimated difference, 26.9%; 95% confidence interval [CI], 13.5-40.4; $P < .001$). The treatment effect was consistently in favor of anastrozole, regardless of the measurement methods (caliper and ultrasound). The histopathologic response rate also was better in the anastrozole group (anastrozole plus goserelin, 41.8%; tamoxifen plus goserelin, 27.3%; estimated difference, 14.6%; 95% CI, 1.4-27.7; $P = .032$). Both treatment regimens were well tolerated, consistent with the known safety profiles of anastrozole, tamoxifen, and goserelin.[20] The geometric mean Ki-67 index at baseline was 21.9% in the anastrozole group and 21.6% in the tamoxifen group. At week 24, the Ki-67 index was reduced in both treatment groups (to 2.9% in the anastrozole group and to 8% in the tamoxifen group). The reduction from baseline to week 24 was significantly greater with anastrozole than with tamoxifen. The estimated ratio of reduction between groups was 0.35 (95% CI, 0.24-0.51; $P < .001$).[20] Here, we report an exploratory analysis of the STAGE study that investigated potential correlations between the Ki-67 index and the best overall tumor response, ER status, PgR status, or histopathologic response.

MATERIALS AND METHODS

Study Design and Patients

In this phase 3, double-blind, randomized, parallel-group, multicenter trial, the participating patients were premenopausal women ≥20 years with ER-positive and HER2-negative breast cancer who had operable and measurable lesions (tumors measuring 2-5 cm, negative lymph node status [N0], and no metastases [M0]). Inclusion and exclusion criteria have been described previously.[20]

Patients were randomized 1:1 to receive either oral anastrozole 1 mg daily with a tamoxifen placebo or oral tamoxifen 20 mg daily with an anastrozole placebo. Both treatment groups received goserelin 3.6 mg as a subcutaneous injection every 28 days. Treatment continued for 24 weeks before surgery or until patients met any criterion for discontinuation.

The primary study endpoint was the best overall tumor response during the 24-week neoadjuvant treatment period. Secondary endpoints included histopathologic response, changes in estrone (E_1) and estradiol (E_2) serum and breast tumor tissue concentrations, changes in Ki-67 expression, and tolerability. For this exploratory analysis, we assessed correlations between Ki-67 expression and tumor response, ER status, PgR status, or histopathologic response.

The protocol was approved by an institutional review board at all study sites, and all enrolled patients provided written informed consent. The study (National Clinical Trials identifier NCT00605267) was conducted in accordance with the Declaration of Helsinki and good clinical practice, the applicable local regulatory requirements, and the AstraZeneca policy on Bioethics.

Assessments

Tumor measurements were performed using caliper measurements, ultrasound, or MRI or CT studies. The

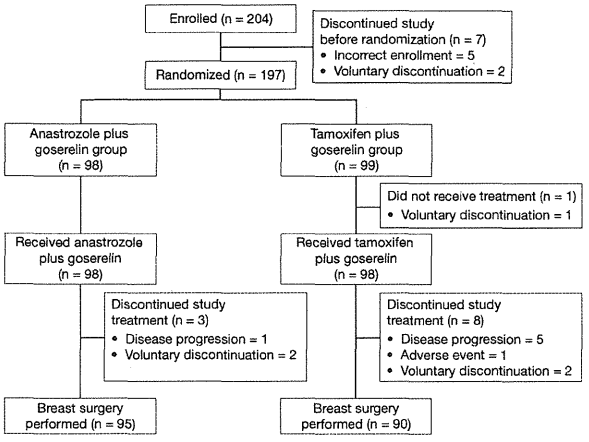

Figure 1. This is a CONSORT (Consolidated Standards of Reporting Trials) diagram of the current study.

primary analysis indicated that the best overall tumor response for anastrozole versus tamoxifen was consistent, regardless of the measurement method used.[20] We present tumor response data from the MRI or CT measurements at day 0 and at 24 weeks. The objective tumor response was assessed according to modified Response Evaluation Criteria in Solid Tumors (RECIST).[21]

The status of Ki-67, ER, and PgR was determined using histopathologic core-needle biopsy specimens that were collected at baseline and at surgery. Tissue sections were fixed in formalin and stored at room temperature before immunohistochemical staining. Ki-67 expression was determined by staining sections with an anti-MIB-1 antibody at a central laboratory (SRL Inc., Tokyo, Japan) for assessment by a central review board. For all slides, photomicrographs were taken from 3 to 5 hotspots at ×20 magnification using light microscopy. Two pathologists independently assessed the photomicrographs, and the Ki-67 index was calculated as the ratio of Ki-67-positive cancer cells from a total of 1000 cancer cells. ER-positive status and PgR-positive status at baseline were defined as ≥10% staining of cancer cell nuclei determined by a pathologist at each individual study site (nuclei were assessed using mouse monoclonal antibody clones 6F11 and 16, respectively). Staining for ER and PgR also was assessed in parallel using Allred scores by the Central Pathologist Review Committee.[22] An Allred score (the proportion score plus the intensity score) of ≥3 defined ER or PgR positivity, a score from ≥3 to <7 indicated medium expression, and a score of ≥7 indicated rich expression.

Histopathologic effects were assessed by comparing histopathologic samples that were obtained at baseline and at surgery. For the assessment of histopathologic response, the following categories were used: grade 0 indicated no response; grade 1a, marked change in <1 of 3 cancer cells; grade 1b, marked changes in ≥1 of 3 but <2 of 3 cancer cells; grade 2, marked changes in ≥2 of 3 cancer cells; and grade 3, necrosis or disappearance of all cancer cells and replacement of all cancer cells by granuloma-like and/or fibrous tissue. The histopathologic response was defined as the proportion of patients whose tumors were classified as grade 1b, 2, or 3.[23,24]

Post hoc subset analyses were used to determine correlations between the baseline Ki-67 index (≥20% vs <20%) and the best overall tumor response. The percentage change in the Ki-67 index for responders (patients whose best overall tumor response was a complete or partial response) versus nonresponders (patients whose best overall tumor response was stable or progressive disease) also was compared. Correlations between the baseline Ki-67 index and the histopathologic response at week 24 also were evaluated, and we used post hoc analyses to investigate correlations between changes in the Ki-67 index from baseline to week 24 and ER or PgR status at baseline. Positive ER and PgR status (Allred score ≥3) also was assessed at baseline and at week 24. Preoperative Endocrine Prognostic Index (PEPI) scores, which were calculated post hoc as the sum of risk points weighted by the size of the hazard ratio for tumor size, pathologic lymph node status, ER status, and Ki-67 expression for both recurrence-free and breast cancer-specific survival, were determined for each patient at surgery according to the methods described by Ellis and colleagues.[25]

Statistical Analysis

The sample size calculation and the main statistical analyses have been described previously.[20] All randomized patients were included in the intent-to-treat analysis set.

In a post hoc exploratory analysis, chi-square tests were performed to compare the best overall tumor response at week 24 between baseline Ki-67 index categories (≥20% vs <20%) within each treatment group and between treatment groups within each baseline Ki-67 index category. A chi-square test also was used to compare the histopathologic response at 24 weeks between the baseline Ki-67 index categories within each treatment group. All tests were made at the nominal 2-sided significance level of .05.

RESULTS

Patients

In total, 197 patients were randomized to receive either anastrozole plus goserelin (n = 98) or tamoxifen plus goserelin (n = 99) (Fig. 1). Patient demographics and