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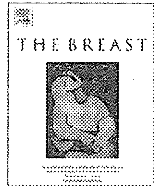

Original article

Sentinel and nonsentinel lymph node assessment using a combination of one-step nucleic acid amplification and conventional histological examination

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ABSTRACT

Background: Clinical significance of intraoperative sentinel lymph node (SLN) metastases detection using one-step nucleic acid amplification (OSNA) has not been thoroughly investigated. The aim of this study was to assess the usefulness of using a combination of OSNA and conventional histological examinations.
Materials and methods: We included 772 consecutive patients with clinical node-negative cTis-cT3 primary breast cancer who underwent SLN biopsy with intraoperative OSNA and multi-section histological examination at our institution. We estimated the concordance rate and compared SLN metastases detection rates between the two methods. We also compared non-SLN metastasis detection rate between patients who tested positive in OSNA and those who tested positive in histology.
Results: Among 772 patients, SLN metastases were intraoperatively detected in 211 (26.4%) by either OSNA or histology, in 168 (21.8%) by OSNA, and in 150 (19.4%) by histology. The concordance rate between OSNA and histological examination was 89.2%, but only 123 (58.8%) patients tested positive in both OSNA and histology; 45 were positive in OSNA only and 43 were positive in histology only.
SLN status as per both OSNA and histology was significantly correlated with the presence of non-SLN metastases and multivariate analysis-identified independent predictive factors of non-SLN metastases.
Conclusions: Intraoperative SLN metastases detection may be more accurate with a combination of OSNA and histological examination than with OSNA or histological examination alone. By using both methods, we can reduce the risk of false negative rate in SLN biopsy, and may prevent physicians from overlooking patients with non-SLN metastases.

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Introduction

Conventional intraoperative histological examinations in sentinel lymph node (SLN) biopsy are well known to show high (10–30%) false-negative results for metastatic foci because only a few thin sections from a lymph node are examined in this technique. The suboptimal quality of frozen section slides and oversights by pathologists increase the false-negative detection rate. Moreover, use of more intensive methods, such as serial-step section examination of each SLN, is impractical because it requires a heavy workload for pathologists [1].

Molecular assays have been developed to overcome these shortcomings. The one-step nucleic acid amplification (OSNA) assay (Sysmex, Kobe, Japan), which involves amplification and quantitative measurement of cytokeratin 19 (*CK19*) mRNA levels, can detect lymph node metastases as accurately as can conventional histological examination, is faster [2], and detects more low-volume tumor nodal involvement than do conventional histological methods. However, whether these techniques can verify the need for further axillary treatment is unclear [3].

This study compared detection rates between OSNA and histological examination, both for intraoperative SLN metastases and for non-SLN metastases. We also discuss the possibility of omitting axillary lymph node dissection (ALND) for some patients with positive SLN metastases (SLN$^+$)—specifically, those histological micrometastases or isolated tumor cells (ITC), and OSNA 1^+ patients.

Materials and methods

Subjects comprised 772 consecutive patients with clinically node-negative Tis-T3 primary breast cancer who underwent SLN

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biopsy with combined intraoperative OSNA and histological examination between February 2010 and June 2012 at the National Cancer Center Hospital, Tokyo, Japan. Patients who received neoadjuvant therapy, and male patients were excluded.

Clinical and pathological T and N factors were based on the Cancer Staging Manual of the American Joint Committee on Cancer (AJCC), 7th edition [4]. Patient characteristics are listed in Table 3. The cut-off value for ER and PR positivity was 10% positive cells for both, irrespective of intensity. HER2 positivity was defined as an HER2 score of >3 (>30% strong membrane immunoreaction-positive cells) or an HER2 gene/centromere 17 ratio of ≥2.0 as assessed by fluorescence in situ hybridization.

SLN biopsy procedure

First, 0.1 ml of 99mTc-phytate was prepared. Half of this solution was injected into the dermis of the areola while the remainder was injected into the dermis over the tumor on the day before surgery. In all patients, lymphoscintigraphy was performed 3 h after injection. In addition, 3–4 ml blue dye or 1 ml indocyanine green was injected into the peritumoral space or areola at the time of surgery. SLNs were identified using a hand-held gamma probe guided by nodal staining. Nodes that responded to near-infrared light, were stained with blue dye, or had high radioactive count were considered as SLNs. No more than four resected SLNs per patient were intraoperatively evaluated by both OSNA and histological examination. We omitted axillary dissection in patients with no SLN metastases and performed axillary dissection in patients with histological macrometastases, micrometastases, or ITCs in SLNs or positivity in OSNA. These patients were considered SLN^+ in this study.

Preparation of SLNs

Excised SLNs were cut into 2-mm slices along the short axis and were alternately prepared for OSNA and histological examination.

Histological examination of SLNs

The sliced tissue specimens for histological examination were first subjected to intraoperative frozen-section diagnosis. These sliced tissues were then fixed in 10% formalin overnight, embedded in paraffin, cut into 4-μm-thick sections, stained with hematoxylin and eosin (HE), and subjected to permanent-section diagnosis.

Macrometastases were defined as SLN^+ that measured >2 mm in greatest diameter, micrometastases as SLN^+ that measured 0.2–2 mm in greatest diameter, and ITC as small clusters of cells ≤0.2 mm across their greatest diameter, as detected by HE staining or immunohistochemistry. Although ITCs are recommended to be classified as $pN0(i^+)$, they were considered as metastases in the present study.

OSNA assay for SLN examination

The details of the OSNA assay based on the RT-LAMP method were previously described by Tsujimoto et al. [5] Briefly, resected SLNs were homogenized with 4 ml lysis buffer solution and centrifuged at 10000× g at room temperature. The RD-100i system (Sysmex, Kobe, Japan) was used to analyze 2 μl of the lysed SLN supernatant.

Using OSNA, SLNs were considered to be SLN^- when the CK19 mRNA copy number was $<2.5 \times 10^2$/μl, SLN^+ 1^+ when the copy number was 2.5×10^2/μl–5.0×10^3/μl, and metastases-positive 2^+ when the copy number was $>5.0 \times 10^3$/μl. A 1:10 dilution of homogenized lymph node solution was always prepared for each patient and analyzed simultaneously because excess protein may interfere with the RT-LAMP reaction [5]. Lymph node lysates showing >250 copies/μl of CK19 mRNA only in the 1:10 diluted solution were classified as positive and designated as +I (inhibition positive). Permanent histological tissue sections were immunostained for CK19 when samples that were SLN^- by OSNA were histologically SLN^+.

Permanent histological examination of non-SLNs

ALND was performed when specimens were SLN^+ by either OSNA or histology. All non-SLNs were bisected along the long axis after formalin fixation. For each non-SLN, only one permanent HE tissue section for the representative cut surface was histologically examined.

Statistical analysis

We used the Mann–Whitney test to compare age and BMI between SLN^+ and SLN^- patients, the χ^2 test to compare other variables, and performed logistic regression analysis to investigate odds ratios of individual parameters for non-SLN metastases. $P < 0.05$ was considered statistically significant. Confidence intervals (CIs) were set at the 95% level. SPSS statistical software (version 19, IBM SPSS Statistics, Chicago, IL, USA) was used for all statistical analyses.

Results

Concordance rate between histology and OSNA

SLN metastases, including ITC, were detected in 211 (27.3%) of the 772 patients: 145 (18.8%) by intraoperative examination of frozen HE-stained sections, 168 (21.8%) by OSNA, and 166 (21.5%) by the examination of permanent HE-stained sections (Table 1). Because we regarded ITC as histological metastases, ALND was performed for patients with ITC in SLNs.

The concordance rate between OSNA and intraoperative histological diagnosis was 88.2%, and that between OSNA and permanent histological diagnosis was 88.6%. The kappa value between OSNA diagnosis and permanent histological diagnosis was 0.66, indicating substantial concordance.

Table 1
Concordance of sentinel lymph node metastasis between OSNA diagnosis and histological diagnosis.

A. Comparison with frozen section diagnosis

	Number of patients (%)		
	Total	Intraoperative frozen section	
		Histology (+)	Histology (−)
OSNA (+)	168	111	57
OSNA (−)	604	34	570
Total	772	145	627

B. Comparison with frozen section + permanent section diagnosis

	Number of patients (%)		
	Total	Intraoperative + permanent sections	
		Histology (+)	Histology (−)
OSNA (+)	168	123	45
OSNA (−)	604	43	561
Total $\kappa = 0.66$	772	166	606

Histology (+) includes macrometastasis, micrometastasis, and isolated tumor cells (ITC), whereas Histology (−) includes others. OSNA (−) includes OSNA 2+, 1+, and +I, whereas OSNA (−) includes OSNA-.

Table 2
Detailed comparison of OSNA results with histological results for sentinel lymph node metastasis.

OSNA	Number of cases (%)				
	Total	Histological diagnosis (permanent section)			
		Macromestasitasis	Micrometastasis	ITC	Negative
2+	90	78 (87)	8 (9)	0 (0)	4 (4)
1+	72	16 (22)	15 (21)	5 (7)	36 (50)
+I	6	1 (17)	0 (0)	0 (0)	5 (83)
−	604	9 (1)	23 (4)	11 (2)	561 (93)
Total	772	104	46	16	606

Among the 168 OSNA$^+$ patients, SLN metastases were histologically detected in 123 (73%), including five with ITC. The remaining 45 (27%) patients were histologically SLN$^-$ (Tables 1 and 2). When OSNA results were stratified, SLN$^+$ rate per permanent histological examination was 96% (86 of 90) for OSNA 2 + patients, 50% (36 of 72) for OSNA 1 + patients, and 17% (one of six) for OSNA + I patients. Among the 604 OSNA$^-$ patients, 43 (7%) were histologically SLN$^+$.

In contrast, 123 (74%) of the 166 histologically SLN$^+$ patients, including 16 with ITC in SLNs, were SLN$^+$ using OSNA.

Clinicopathological correlation with SLN status by histology and OSNA

Clinicopathological characteristics of the 772 patients are listed in Table 3. For SLN statuses detected by both permanent histology and OSNA, SLN metastases significantly correlated with cT-factor, pT-factor, histological type, LVI (lymphovascular invasion) and histological grade.

There were no significant differences in characteristics between patients with SLN metastases detected by OSNA and histological

Table 3
Correlations of clinicopathological parameters with sentinel lymph node status, detected by histopathological examination and by OSNA method.

Parameter	Number of cases (%)						
	Total N = 772	SLN status (%)			SLN status (%)		
		Histology (+)N = 166	Histology (−)N = 606	p	OSNA (+)N = 168	OSNA (−)N = 604	p
Age							
Average (range)	56.3 (27–92)	54.9 (27–84)	56.8 (28–92)	NS	54.1 (27–92)	56.9 (28–92)	NS
<50	256	59 (23)	197 (77)		64 (25)	191 (75)	
≧50	516	107 (21)	409 (79)		104 (20)	413 (80)	
Menopause							
Premenopausal	311	72 (23)	239 (77)	NS	78 (25)	233 (75)	NS
Postmenopausal	457	94 (21)	363 (79)		90 (20)	367 (80)	
Unknown	4	0	4 (100)		0	4 (100)	
BMI							
Average (range)	22.1 (13.2–40)	22.6 (17–40)	22.0 (13.2–35.8)	NS	22.1 (17–40)	22.1 (13.2–35.8)	NS
<25	645	138 (21)	507 (79)		147 (23)	498 (77)	
≧25	126	28 (22)	98 (78)		21 (17)	105 (83)	
Unknown	1	0 (0)	1 (100)		0 (0)	1 (100)	
CT-factor							
Tis	159	6 (4)	153 (96)	<0.0001	8(5)	151 (95)	<0.0001
T1	355	66 (19)	289 (81)		76 (21)	279 (79)	
T2	252	90 (36)	162 (64)		80 (32)	172 (68)	
T3	6	4 (67)	2 (33)		4 (67)	2 (33)	
PT-factor							
Tis	119	0 (0)	119 (100)	<0.0001	8 (7)	111 (93)	<0.0001
T1	413	72 (17)	341 (83)		73 (18)	340 (82)	
T2	209	73 (35)	136 (65)		68 (33)	141 (67)	
T3	29	21 (72)	8 (28)		19 (66)	10 (34)	
Unknown	2	0 (0)	2 (100)		0 (0)	2 (100)	
His to logical type							
Carcinoma in situ	119	0 (0)	119 (100)	<0.0001	8 (7)	111 (93)	<0.0001
Invasive ductal	566	150 (27)	416 (73)		143 (25)	423 (75)	
Special	86	16 (19)	70 (21)		17 (20)	69 (80)	
Others	1	0	1 (100)		0 (0)	1 (100)	
Lymphovascular invasion							
Negative	533	65 (12)	468 (88)	<0.0001	74 (14)	459 (86)	<0.0001
Positive	230	101 (44)	129 (56)		92 (40)	138 (60)	
Unknown	9	0 (0)	9 (100)		2 (22)	7 (78)	
Histological grade							
1	217	17 (8)	200 (92)	<0.0001	26(12)	191 (88)	<0.0001
2	351	95 (27)	256 (73)		84 (24)	267 (76)	
3	202	54 (27)	148 (73)		58 (29)	144 (71)	
Unknown	2	0 (0)	2 (100)		0	2 (100)	
Hormone receptor							
Negative	113	19 (17)	94 (83)	NS	21 (19)	92 (81)	NS
Positive	658	146 (22)	512 (78)		146 (22)	512 (78)	
Unknown	1	1 (100)	0 (0)		1 (100)	0 (0)	
HER2							
Negative	671	148 (22)	523 (78)	NS	147 (22)	524 (88)	NS
Positive	86	17 (20)	69 (80)		18 (21)	68 (89)	
Unknown	15	1 (7)	14 (93)		3 (20)	12 (80)	

Histology (+) includes macrometastasis, micrometastaas, and isolated tumor cells (ITC), whereas Histology (−) includes others. OSNA (+) includes OSNA 2+, 1+, and +I, whereas OSNA (−) includes OSNA−.

method. However, despite no statistical significance, percentages of early and low-grade tumors tended to be larger in the former group: no patient with carcinoma in situ showed histological SLN metastases, whereas eight (7%) of 119 patients with carcinoma in situ showed positivity in OSNA. Similarly, LVI^- (14% vs 12%), and histological grade 1 (12% vs 8%) tumors tended to test positive more frequently in OSNA (Table 3).

Correlation of SLN status with non-SLN status in patients who underwent ALND

Among the 211 SLN^+ patients, 206 underwent ALND after SLN biopsy. The correlation of SLN status with non-SLN status based on OSNA and permanent histological examination is summarized in Table 4.

Among the 206 SLN^+ by OSNA or histology, 53 (26%) had non-SLN metastases. The overall incidence of non-SLN metastases among patients with histological SLN metastases was 32% (52 of 162): 40% (42 of 104), 20% (nine of 44), and 8% (one of 13) for patients with macrometastases, micrometastases, and ITC in SLNs, respectively. In contrast, only one (2%) of the 44 patients with $OSNA^+$ but $histology^-$ SLN metastases exhibited non-SLN metastases (Table 4).

Clinicopathological characteristics were analyzed in a multivariate logistic regression model. Histological SLN status (macrometastases/nonmalignant cells: odds ratio, 12.17; 95% confidence interval (CI); 1.45–102.34; $P = 0.020$) and OSNA-determined SLN status ($OSNA2^+$ to OSNA−: odds ratio, 4.75; 95% CI, 1.23–17.35; $P = 0.018$) were identified as independent predictive factors for non-SLN metastases (Table 5). These data indicate that OSNA 1^+ status was not an independent predictor for non-SLN metastases.

Discussion

The concordance rate of SLN metastasis detection between OSNA and histological diagnoses is reportedly high, ranging from 86.3% to 96.3% [1,2,7–14]. The SLN metastasis detection rate by OSNA was higher than that by histological examination because OSNA can detect tumor cells in whole tissues [14–16].

Table 4
Comparison between sentinel lymph node (SLN) status and non-SLN status.

SLN status			Number of cases (%)			
			Total	Non-SLN metastases		
pN stage	Histology	OSNA		Positive	Negative	Subtotal
PN1	Macrometastasis	2+	78	37 (47)	41	42/104 (40)
		1+	16	4 (25)	12	
		+I	1	0 (0)	1	
		–	9	1 (11)	8	
PN1mi	Micrometastasis	2+	8	1 (13)	7	9/45 (20)
		1+	15	5 (33)	10	
		–	22	3 (14)	19	
pN0(i+)[a]	ITC	2+	0	0 (0)	0	1/13 (8)
		1+	5	1 (20)	4	
		–	8	0 (0)	8	
pN0 (mol+)[b]	–	2+	4	1 (25)	3	1/44 (2)
		1+	35	0 (0)	35	
		+I	5	0 (0)	5	
			206	53	154	53/206 (26)

Five patients who did nor receive ALND were excluded from the calculation.
[a] Axillary macro- or micrometastases absent but ITC present as per histology, regardless of OSNA results.
[b] Axillary metastases absent as per histology but present as per OSNA (2+, 1+, and/or + I).

Table 5
Predictive factors for non-SLN metastasis by multivariate logistic regression model analysis.

Parameter	Odds Ratio	95% confidence interval	p Value
pT factor			
PT1	1		
pT2	1.29	0.59–2.83	0.52
pT3	2.64	0.89–7.85	0.081
Hormone receptor status			
Positive	1		
Negative	2.47	0.88–6.96	0.088
SLN status by histology			
No malignant cell	1		
ITC	4.99	0.26–95.17	0.285
Micrometastasis	8.98	0.99–81.76	0.052
Macrometastasis	12.17	1.45–102.34	0.02
SLN status by OSNA			
–	1		
1+	2.27	0.59–8.66	0.232
2+	4.75	1.23–17.35	0.018

We did not include patients with pTis and OSNA + I in the analysis because there were no patients with non-SLN metastases in these groups.

However, histological corroboration of cancer volume is impossible if entire SLN tissues are used for OSNA assays. We considered that comparison of intraoperative OSNA results with intraoperative histology results was necessary for several patients before complete substitution of intraoperative histological diagnosis by OSNA. In this study, the SLN metastasis detection rate using combined OSNA and histology was 27.1% higher than that by histology only and 25.6% higher than that by OSNA only.

Among the SLN^+ 211 patients using either method, 88 showed discordant results. Only 123 (58.3%) patients were both $OSNA^+$ and $histology^+$. Such discordances were especially common among patients who were OSNA 1^+ (50%, 36 of 72), OSNA + I (83%, five of six), and those with $histology^+$ micrometastases (50%, 23 of 46) and $histology^+$ ITC (69%, 11 of 16) in SLNs. In contrast, the discordance rate was only 4% (four of 90) among OSNA 2^+ patients and 9% (nine of 104) among patients with $histology^+$ macrometastases.

Reportedly, most discrepancies occur because of uneven distribution of minuscule metastases [6,7]. Vegue et al. showed that histological examination of a single SLN section misclassified 41.8% patients as SLN^- compared OSNA data ($P = 0.007$) [17]. Although we histologically assessed multiple slices of SLN samples (2-mm intervals, two to seven slices per node) to ensure accurate comparison of SLN^+ rates between the two methods, uneven distribution of metastatic foci in SLNs appeared to occur in >40% SLN^+ patients. Tamaki et al. examined SLN metastases using both OSNA and histology methods similar to those used in the present study and reported that discordant results due to uneven distribution occurred in 38% of patients with $OSNA^+$ SLNs and 11% of patients with $histology^+$ SLNs [7].

Another possible explanation for this discordance may lie in the false-negative results exhibited by tumors with low CK19 expression. Low CK19 protein expression is reported in approximately 2–3% of breast cancers [6,18]. In the present study, we performed CK19 immunostaining for 16 of 31 patients with $histology^+$ but $OSNA^-$ tumors; however, we found that only one of 16 patients with positive residual SLN metastases was $CK19^-$. Therefore, most discordant results were attributed to uneven distribution of tumor cell foci in each SLN.

In the present study, the non-SLN metastasis detection rate was high in OSNA 2^+ patients (43%, 39 of 90) and patients with SLN micrometastases (40%, 42 of 104). In addition, the incidences of non-SLN metastases among OSNA 1^+ patients and patients

with histology$^+$ micrometastases were 14% (10 of 71) and 20% (nine of 45), respectively. The present data concords with those reported by Castellano et al., in which OSNA 2^+ patients had a 42% chance of non-SLN metastases while OSNA 1^+ patients had a 22% chance of non-SLN metastases [6]. When both OSNA and histology were combined, the rate of non-SLN metastasis detection was extremely low in patients with histology$^+$ ITC in SLNs (regardless of OSNA status; 8%; 1 of 13), OSNA 1^+ patients without histological metastases (0%; 0 of 35), and OSNA + I patients without histological metastases (0%; 0 of five). Both histology$^+$ metastasis size and semiquantitative OSNA SLN data were significant independent predictors of non-SLN metastases according to logistic regression analysis. Moreover, combined use of OSNA and histological examination could identify patients whose SLN statuses, – specifically, ITC, OSNA 1^+, and OSNA + I, without histology$^+$ tumor deposits –, correlated with low risk of non-SLN metastasis.

Non-SLN metastases would have been overlooked in maximum 10% (four of 39) of SLN$^-$ patients if OSNA alone had been used. Similarly, non-SLN metastases would have been overlooked in 2% (one of 44) of SLN$^-$ patients if histological examination alone had been used. These estimations imply that ALND can be omitted in patients with SLN$^+$ detected by OSNA only in combined OSNA and histological examination. The non-SLN metastasis detection rate was much lower in histology$^-$ SLNs than for OSNA$^-$ SLNs when combined examination was used.

OSNA tended to detect SLN metastases more frequently than histological examination in primary tumors with non-invasive histology, histological grade 1, and lack of LVI. Although Osako et al. reported that OSNA could detect metastases more frequently than histological frozen-section examination in elderly or postmenopausal patients [15], we could not find such an interaction on using OSNA.

In the present study, there were 21 discordant diagnoses between intraoperative frozen section examination and permanent section examination. These included three cases of macrometastases, 11 of micrometastases, and seven of ITC. OSNA detected more than half of the metastases missed by frozen section diagnosis: two of three cases of macrometastasis samples, six of 11 cases of micrometastasis, and four of seven cases of ITC samples. An advantage of using the OSNA assay is that it confirms histological results and identifies patients who require ALND. However, OSNA may also lead to unnecessary ALNDs. Given these circumstances, combining OSNA and histology can prevent physicians from overlooking SLN and non-SLN metastases that can be missed when either method is used alone.

ALND was recently shown to have no significant influence on clinical outcomes of patients with micrometastases or ITC [19,20]. Osako et al. showed that routine histological examination of non-SLN metastases could overlook many occult metastases that can be detected by combined OSNA and histological examination [21]. Our previous study found that SLN and non-SLN occult metastases that were not detected routinely but detected by serial-step sections at 85-μm intervals did not have significant prognostic implications [22].

The OSNA assay is a promising alternative or additional tool for intraoperative detection of SLN metastases. Because of the low rate of metastases to non-SLNs, ALND may be omitted in patients with OSNA 1^+/histology$^-$ SLNs or OSNA$^-$/histology$^+$ ITC$^+$ SLNs when OSNA and histological examination are combined.

To date, however, there is no evidence of whether or not metastases evaluated only by molecular analysis require ALND. Further data on tumor recurrence and patient survival will clarify how SLN metastases detected by molecular methods can be optimally managed.

Conclusions

Intraoperative SLN metastasis detection may be more accurate using a combination of OSNA and histological examination than with OSNA or histological examination alone. This combination technique may prevent physicians from overlooking patients with non-SLN metastases. Although stratification of non-SLN$^+$ and non-SLN$^-$ patients according to the present OSNA categories (2^+, 1^+, and +I) is not perfect, more complete predictions of non-SLN metastases using OSNA may only be possible if stratification of these categories is improved in the near future.

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Conflict of interest statement

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FULL PAPER

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Molecular detection of lymph node metastasis in breast cancer patients treated with preoperative systemic chemotherapy: a prospective multicentre trial using the one-step nucleic acid amplification assay

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Background: For patients with breast cancer treated with preoperative chemotherapy, residual tumour burden in lymph nodes is the strongest prognostic factor. However, conventional pathological examination has limitations that hinder the accurate and reproducible measurement. The one-step nucleic acid amplification (OSNA) assay is a novel molecular method for detecting nodal metastasis. In this prospective multicentre trial, we assessed the performance of the OSNA assay in detecting nodal metastasis after chemotherapy.

Methods: In total, 302 lymph nodes from 80 breast cancer patients who underwent axillary dissection after chemotherapy were analysed. Each node was cut into two or four slices. One piece or alternate pieces were evaluated by pathology, and the other(s) were examined using the OSNA assay. The results of the two methods were compared. Stromal fibrosis, histiocytic aggregates, and degenerated cancer cells were regarded as chemotherapy-induced histological changes.

Results: The overall accuracy, sensitivity, and specificity of the OSNA assay compared with the reference pathology were 91.1%, 88.3%, and 91.7%, respectively. Of the 302 lymph nodes, 66 (21.9%) exhibited chemotherapy-induced histology. For these nodes, the accuracy, sensitivity, and specificity were 90.9%, 88.9%, and 93.3%, respectively.

Conclusion: The OSNA assay can detect the residual tumour burden as accurately as conventional pathology, although chemotherapy-induced histological changes are present.

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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

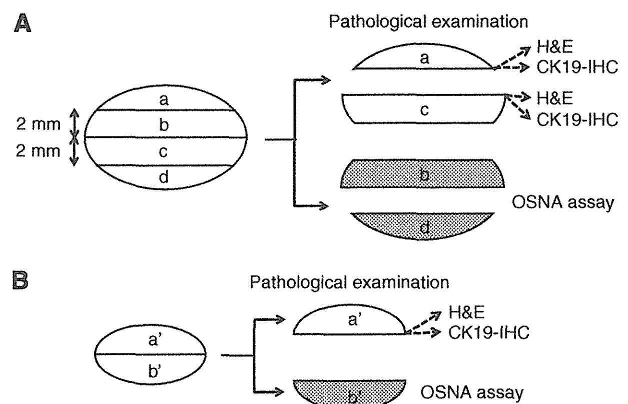

Figure 1. **Lymph node examination process.** Lymph nodes larger than 4 mm were sliced into four pieces (**A**), and lymph nodes sized 4 mm or less were cut into two pieces (**B**). H&E, haematoxylin-and-eosin staining; CK19-IHC, cytokeratin 19 immunohistochemistry; OSNA, one-step nucleic acid amplification.

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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.

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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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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 $\leq$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 $\leq$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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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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