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A Novel Automated Assay for the Rapid Identification of Metastatic Breast Carcinoma in Sentinel Lymph Nodes

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Abstract

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CONFLICT OF INTEREST DISCLOSURES

Sysmex America, Inc. provided funding for the study.

BACKGROUND—The authors prospectively evaluated the performance of a proprietary molecular testing platform using one-step nucleic acid amplification (OSNA) for the detection of metastatic carcinoma in sentinel lymph nodes (SLNs) in a large multicenter trial and compared the OSNA results with the results from a detailed postoperative histopathologic evaluation (reference pathology) and from intraoperative imprint cytology (IC).

METHODS—In total, 1044 SLN samples from 496 patients at 11 clinical sites were analyzed. Alternate 1-mm sections were subjected to either detailed histopathologic evaluation with hematoxylin and eosin and pancytokeratin immunostaining or the OSNA Breast Cancer System, which was calibrated to detect tumor deposits >0.2 mm by measuring cytokeratin 19 messenger RNA. At 7 sites, IC was performed before permanent section. The OSNA results were classified as negative (<250 copies/μL), micrometastases (from ≥250 to <5000 copies/μL), or macrometastases (≥5000 copies/μL).

RESULTS—The sensitivity and specificity of the OSNA breast cancer system compared with reference pathology were 77.5% (95% confidence interval, 69.7%-84.2%) and 95.8% (95% confidence interval, 94.3%-97.0%), respectively, before discordant case analyses (DCA). Sensitivity and specificity after DCA were 82.7% and 97.7%, and final concordance was 95.8%. Performance for invasive lobular carcinoma demonstrated 88.2% sensitivity (95% confidence interval, 63.6%-98.5%) and 98.5% specificity (95% confidence interval, 92%-100%). The sensitivity of OSNA was significantly better than that of IC (80% vs 63%; *P* =.0229).

CONCLUSIONS—The OSNA breast cancer system proved to be highly accurate for the detection of metastatic breast cancer in axillary SLNs. Sensitivity was comparable to that predicted for conventional postoperative histologic examination at 2-mm intervals and was significantly more sensitive than IC. Automation, semiquantitative results enabling the differentiation of macrometastasis and micrometastasis, and rapid results render the assay suitable for intraoperative and/or permanent evaluation of SLNs.

Keywords

breast; carcinoma; sentinel; lymph nodes; nucleic acid amplification; OSNA

Lymphatic mapping with the identification and removal of axillary sentinel lymph nodes (SLNs) currently is standard practice in the surgical management of patients with early stage breast cancer.[1-3] SLNs are accurate predictors of the status of nonsentinel lymph nodes, with a negative predictive value (NPV) approaching 100%.[4] Intraoperative evaluation of SLNs allows for complete axillary dissection at the time of primary breast surgery if the SLNs are identified as positive for metastatic tumor, eliminating the need for a second surgical procedure with its associated costs, morbidity, and patient distress.

Although there is general agreement that SLNs should be sliced at 2-mm intervals for pathologic evaluation, to our knowledge, no consensus exists regarding the optimal method for intraoperative and final pathologic examination of SLNs in breast cancer. Intraoperative evaluation may include frozen section, touch imprint cytology (IC), scrape cytology, or a combination of these techniques. Although the specificity of these techniques is excellent, the sensitivity varies widely, ranging from 50% to 75%.[5-8] It has been reported that intraoperative frozen section analysis of the entire lymph node sectioned at 50-μm intervals

dramatically increases the sensitivity of detecting metastatic disease, although it is not feasible or practical at most centers.[4] Intraoperative rapid cytokeratin immunostaining also can improve the sensitivity of the evaluation[9]; however, this test is not widely available. There also is much variability in the protocols adopted by different laboratories for the final pathologic evaluation of SLNs in breast cancer. In addition, pathologic assessments examine a very small amount of the lymphoid tissue and are subject to interobserver variability in interpretation,[10] prompting the development of standardized techniques.

Molecular testing of SLNs can enable standardized, objective, and rapid evaluation. In this report, we describe a novel, fully automated molecular device, the "One Step Nucleic Acid Amplification (OSNA) Breast Cancer System" (Sysmex Corporation, Kobe, Japan), which combines reverse transcription (RT) with isothermal loop-mediated DNA amplification (RT-LAMP) for the detection of cytokeratin 19 (CK19) messenger RNA (mRNA) as a marker of metastatic carcinoma in SLNs. In a large, prospective, multicenter study, we compared the performance of the OSNA system with that of a detailed histopathologic examination of the lymph node and with IC for the detection of metastatic carcinoma in axillary SLNs in patients who had early stage breast cancer.

MATERIALS AND METHODS

Study Population

The study was conducted prospectively at 11 US clinical sites after institutional review board approval was obtained. Patients aged >18 years with clinical tumor in situ (Tis), T1, or T2 primary breast cancer who were awaiting lymphatic mapping and SLN biopsy were eligible for enrollment. Exclusion criteria included locally advanced breast cancer (tumors classified as T3 or T4), ductal carcinoma in situ in patients who were undergoing breast-conserving surgery, clinically palpable suspicious axillary lymph nodes, previous diagnosis of another type of carcinoma, previous breast or axillary surgery, and pre-operative neoadjuvant therapy.

SLN Sectioning and Central Pathology Review

Lymphatic mapping and SLN biopsy were performed using standard techniques with blue dye and/or technetium 99m (^{99m}Tc) sulfur colloid. SLNs that measured 4 mm to 20 mm along the long axis with a thickness that ranged from 4 mm to 10 mm were included. All 496 patients had at least 1 lymph node that was evaluable by both test methods. SLNs were cut using a proprietary, 5-blade lymph node cutter with an interblade distance of 1 mm, which sectioned the SLNs into an average of 6 pieces along the long axis. Although the central pieces were cut uniformly into 1-mm slices, the edges could be ≥2 mm in thickness, in which case, they were manually bisected. Alternate slices of the lymph node were subjected either to analysis with the OSNA system or to detailed histopathologic examination. For patients who had an intraoperative evaluation (7 centers), IC was performed on all slices or on the slices that were selected for the reference method. Slices of the SLNs that were selected for histopathology were fixed in formalin and embedded in paraffin. Pathologists at the individual clinical sites evaluated the SLNs according to the standard protocol established at each site for clinical management. Paraffin blocks of the

SLNs subsequently were cut at 200-μm intervals (levels) until all tissue was depleted. At each level, three 5-μm sections were cut; the first section for each level was stained with hematoxylin and eosin (H&E), and the third section from the third level was stained immunohistochemically using pan-CK antibodies. The remaining sections were blanks to be used for additional staining, if needed. All slides, including the H&E-stained, pan-CK-immunostained, and blank sections, were sent to a central reference pathology laboratory (Quest Diagnostics, Terterboro, NJ) for evaluation by at least 2 independent pathologists who were blinded to the histopathology results from the clinical sites and the results from the OSNA system. Tumor deposits in the SLNs were classified according to American Joint Committee on Cancer guidelines.[11]

The OSNA Breast Cancer System

The OSNA Breast Cancer System (Sysmex Corporation) consists of an automated gene-amplification analyzer and homogenization and gene-amplification buffers and controls to detect CK19 mRNA, which is an established epithelial cell marker for the detection of metastatic carcinoma in SLNs.[12] The RT-LAMP method, CK19 target and β-actin internal control primers were described previously.[12-17] The SLN slices that we selected for the assay were homogenized in 4 mL of OSNA lysis buffer and centrifuged according to the manufacturer's directions. A 1:10 dilution of the RNA-rich middle layer was transferred into the analyzer, which automatically performed the amplification reaction and analysis. The device was calibrated to designate samples that contained ≥250 copies per μL of CK19 mRNA as positive for metastatic tumor. Cutoff values, system calibration, and calculation of the CK19 mRNA level of the sample from the calibration curve were determined as described previously.[12] A negative control was analyzed during the calibration and sample analysis to check for contamination issues, and a positive control was analyzed to check for any reagent quality or instrument issues.

Data Analysis

Standard performance characteristics, including sensitivity, specificity, and agreement as well as the positive predictive value (PPV) and the NPV using exact binomial 95% confidence intervals, were calculated to compare the OSNA system with histopathology. Study design and all sample size calculations were determined on an SLN basis; nevertheless, performance characteristics calculated on a per patient basis did not yield significantly different results.

Discordant Case Analyses

Discordant results could be caused by tissue allocation bias, in which metastasis is present only in the tissue specimen being analyzed by 1 of the 2 methods. Specimens with discordant results were evaluated by using multiple techniques. Blank tissue sections were stained with CK19-specific antibody; back-up samples were retested with the OSNA system multiple times to check for operator errors; Western blot analysis of CK19 protein and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of CK19, SAM-pointed domain-containing ets transcription factor (SPDEF), and forkhead box A1 (FoxA1) gene expression were performed.[12]

RESULTS

Patient Demographics

We sampled 1044 SLNs from 496 patients who were enrolled at 11 clinical sites, including 4 academic and 7 community hospitals (175 patients and 321 patients, respectively) who met the study inclusion criteria and had valid results with the OSNA system and with reference pathology. Patient demographics are provided in Table 1.

OSNA System Performance

A performance evaluation of the OSNA system compared with reference pathology is provided in Tables 2 and 3. In this study, 138 of 1044 SLNs (13.2%) obtained from 101 of 496 patients (20.4%) were positive for metastatic disease according to central pathology examination (Table 2). Macrometastases comprised 68.8% and micrometastases comprised 31.2% of these metastases. The OSNA system detected 90.5% (86 of 95) of the reference pathology macrometastases as positive, with a result of ++ in 81% (77 of 95 macrometastases) and a result of + in 9.5% (9 of 95 macrometastases). It detected 48.8% (21 of 43) of the reference pathology micrometastases as positive, with a result of ++ in 20.9% (9 of 43 micrometastasis) and a result of + in 27.9% (12 of 43 micrometastasis). The OSNA system also detected 9 additional macrometastases (OSNA++) and 29 micrometastases (OSNA+) that were negative at reference pathology (Table 2). These data yielded agreement of 93.4%, sensitivity of 77.5%, specificity of 95.8%, a PPV of 73.8%, and a NPV of 96.6% (Table 3). The sensitivity and specificity for macrometastases were 81.1% and 98.1%, respectively (Table 3).

Discordant Case Analyses

There were 71 discordant results in the study, including 31 OSNA-negative/reference pathology-positive results and 38 OSNA-positive/reference pathology-negative results, in addition to 2 pathology assessment reversals upon discovery of tissue allocation bias, conferring a discordance rate of 6.8%. Twenty-eight of the discordant results could be resolved by further discordant analysis; it is noteworthy that all 9 discordant positive OSNA ++ results (macrometastases) were resolved (Tables 4 and 5) and were identified as true misses by reference pathology, most likely because of tissue allocation bias. Inclusion of these 9 macrometastases in assay performance calculations yielded a PPV of 100% for an OSNA++ (macrometastasis) result.

There were 29 discordant cases for the OSNA+ (micrometastasis) result. Nine of the 29 micrometastases were identified as true misses by reference pathology upon further analysis. The remaining 20 unresolved OSNA-positive discordant results were from OSNA+ samples with a median CK19 mRNA count of 615 copies per μL, indicating very small cancer deposits close to the assay cutoff level. One of the 9 discordant OSNA-negative/reference pathology-positive (macrometastasis) samples could be resolved by using these methods (Table 5). Tissue allocation bias could not be ruled out for unresolved discordant cases because of measurement thresholds and inherent limitations of the techniques involved. OSNA system performance after correction for discordant sample testing resulted in

sensitivity and specificity of 82.7% and 97.7%, respectively, with final concordance of 95.8% (Table 3).

Comparison With IC Performance

IC was performed on 1-mm sections from 532 SLNs that were obtained from 272 patients from 7 clinical sites and was analyzed intraoperatively, before permanent section or assay by OSNA. Eighty-one SLNs from 58 patients were identified as positive for breast tumor deposits by reference pathology, including 56 macrometastases and 25 micrometastases. Only 51 of these 81 positive SLNs were detected by IC, yielding a sensitivity of 63% (Table 6). A detailed breakdown of results from both IC and reference pathology is given in Table 6. It is noteworthy that IC missed the identification of metastasis in 30 SLNs and, in particular, failed to detect 15 of the 56 SLNs (26.8%) that had macrometastases identified by reference pathology. Therefore, IC demonstrated 63% sensitivity, 99.3% specificity, a PPV of 94.4%, and an NPV of 93.7% for the detection of metastatic foci in breast cancer axillary SLNs in this study (Table 7).

The OSNA system correctly identified 65 of 81 SLNs that were identified as positive for cancer deposits by reference pathology (Table 6). Although IC missed macrometastases in 15 SLNs (Table 6), the same was true for only 4 SLNs by the OSNA system. The OSNA system also detected more micrometastases than IC (13 vs 10 for OSNA vs IC, respectively). Thus, agreement between OSNA and reference pathology was 93.4%, whereas sensitivity was 80.2% (Table 7). OSNA sensitivity increased to 85.5% after further analysis of discordant samples. Both of these values were significantly better than our finding of 63% sensitivity for IC in our prospective comparison with detailed histopathology (Table 7).

Detection of Lobular Type Breast Cancer in Lymph Nodes

The study included 84 SLNs from 40 patients who were diagnosed with invasive lobular carcinoma (Table 1). Of these 84 SLNs, 17 were positive by reference pathology, including 11 macrometastases and 6 micrometastases. The OSNA system detected all 11 macrometastases and 4 of 6 micrometastases as positive (Table 8). Overall, the system demonstrated an agreement of 96.4%, sensitivity of 88.2%, and specificity of 98.5% with a PPV of 97.1% and an NPV of 93.8% for the detection of metastatic lobular carcinoma (Table 3). There were 2 discordant negative results (Table 8) in which both samples were identified as micrometastases by histopathologic evaluation, and there was 1 discordant positive result (OSNA++, histopathology negative), which was resolved through discordant case investigation.

Suitability for Intraoperative Decisions

To demonstrate the suitability of the device for intraoperative decisions, we tested the timing and technical performance of the OSNA system. Timing was calculated from homogenization of the SLNs to analyzer result output. The timing study was performed on 187 SLNs from 92 study patients in 10 centers. For 1, 2, and 3 SLNs, the interquartile mean total times measured were 33.0 minutes, 39.6 minutes, and 45.2 minutes, respectively, and the minimum times reported were 27.9 minutes, 33.1 minutes, and 37.6 minutes,

respectively. A similarly detailed intraoperative frozen-section analysis schema reportedly took from 30 to 40 minutes.[4]

DISCUSSION

In this large, prospective, multicenter study, the performance of a molecular testing platform was compared with a detailed reference histopathology method for the detection of breast cancer deposits in SLNs. The agreement, sensitivity, and specificity of the OSNA system compared with reference pathology were 95.8%, 82.7%, and 97.7% after discordant sample analysis. The performance results did not vary significantly between academic and community centers (data not shown). The sensitivity and specificity for detecting macrometastases were 81.1% and 98.1%, respectively. It is noteworthy that, although the system failed to detect 9 macrometastases and 22 micrometastases that were identified by reference pathology, it detected 9 macrometastases and 29 micrometastases that were identified as negative or as isolated tumor cells by reference pathology. All of those 9 macrometastases were identified as true misses by reference pathology, and recalculation of the assay performance for macrometastasis yielded a PPV of 100% for an OSNA++ result. Nine of the 29 micrometastases were identified as true misses by reference pathology upon discordant case analysis, and all of the remaining 20 micrometastases were close to the assay cutoff value, suggesting that they were small tumor deposits for which tissue allocation bias could not be ruled out. The calculated sensitivity of the SLN pathologic evaluation scheme recommended by the College of American Pathologists, which includes evaluation of 1 H&E-stained level every 2 mm, is 76.4%.[18] Our finding 82.7% sensitivity for the OSNA system after discordant case analysis suggests comparability performance of the OSNA system and conventional histologic examination for detecting metastatic carcinoma in SLNs. It is noteworthy that the OSNA system performance was superior to that of IC, which is one of the more commonly used techniques for intraoperative SLN evaluation in the United States. OSNA was significantly more sensitive and detected more macrometastasis than IC (OSNA sensitivity, 85.5%; IC sensitivity, 63%; P=.0018).

The sensitivity and specificity of the OSNA system in the current large, multicenter study were lower than the values reported in previous smaller studies that used a similar system, in which the sensitivity ranged from 87.5% to 100% and the specificity ranged from 94.7% to 97.1%.[12-14] Significant differences in the study design most likely were the cause of this variation. There were 3 primary differences in our study: evaluation solely of SLNs; slicing at 1-mm intervals rather than at 2-mm intervals, which provided a more detailed histopathologic examination by exhausting all tissue in the block; and large numbers of micrometastases. Thus, the increased probability of tissue allocation bias, together with the increased likelihood of detecting micrometastases in SLNs compared with axillary lymph nodes, most likely contributed to differences in the results.

The other molecular testing platform is the Breast Lymph Node (BLN) Assay (Veridex, LLC, a Johnson & Johnson Company, Raritan, NJ), which has been approved by the US Food and Drug Administration for intraoperative and/or permanent examination of SLNs.[18-20] This platform identifies metastases that measure >0.2 mm by detecting mammaglobin and CK19 mRNA with real-time RT-PCR. This assay reportedly detects 98%

of metastases >2 mm and 88% of metastases >0.2 mm.[19] Although a direct comparison cannot be made, the OSNA system that was used in our multicenter study demonstrated slightly lower sensitivity compared with the BLN assay. This may be because of differences in study design: our lymph nodes were sliced at 1-mm intervals, whereas the BLN assay studies used 2-mm manually sliced intervals. In addition, we performed a more detailed histopathologic examination of all tissues in the paraffin block than was performed in the BLN assay studies, which used only a limited examination of in paraffin tissue block of the slices that were assigned to the reference method. Finally, the ratio of micrometastasis to macrometastasis on a patient basis was much lower in the BLN study (23:94) than in our study (29:72), increasing the likelihood of tissue allocation bias in our study. An important advantage of the OSNA system over the BLN assay is that the OSNA system is semiquantitative and can differentiate between macrometastasis (OSNA++) and micrometastasis (OSNA+). This is important for clinical decision making (both surgically for complete axillary lymph node dissection [ALND]) as well as for patient treatment decision-making.

Discordant results are a significant concern with any molecular assay. A discordance rate of 4.1% (43 of 1044 samples) was observed after an intense investigation of all discordant samples, which revealed that, of the OSNA++ discordant samples that could be resolved, the majority resulted from tissue allocation bias (6 of 9 OSNA++ samples in Table 5). It is understandable that, in any study design in which the tissue allocated to the different arms of the study cannot be evaluated by the same method, the detection rate of abnormalities will be a function of the calculated distribution of the abnormality. Because the lymph nodes were sliced at 1-mm intervals, the likelihood for discordance in the detection of micrometastases would be higher than that for macrometastases. Our finding that the majority of the discordant cases were micrometastases agrees with the expected distribution of metastases across the lymph nodes with a likelihood of detection by either the trial method or the reference method, but not by both methods. In an intraoperative setting, the likelihood of a false-positive result is of concern. For macrometastases, however, all 9 discordant OSNA++ results were identified as true-positive, and 9 of 29 discordant OSNA+ results also were identified as true-positive upon discordant case analysis. Although 20 of the 29 discordant OSNA+ results could not be confirmed with other technologies given the inherent limitations of these molecular methods, the median CK19 copy numbers in these samples were close to the assay cutoff, suggesting that they were very small micrometastases, and this did not rule out the possibility of tissue allocation bias.

The American Society of Clinical Oncology (ASCO) guidelines recommend completion ALND for patients who have breast cancer micrometastases identified in SLNs.[21] However, there is considerable controversy surrounding this issue, and a recent survey of ASCO members suggested that the majority of breast cancer surgeons do not routinely follow these guidelines.[22] Recently, it was reported that, at a median follow-up of 5.9 years, there were no differences in the regional recurrence rates between SLN-positive patients who were randomized to undergo either SLN biopsy alone or SLN biopsy followed by completion ALND on the American College of Surgeons Oncology Group (ACOSOG) Z0011 trial.[23] Those authors suggested that some patients who had 1 or 2 positive SLNs may not need to undergo completion ALND. The ACOSOG Z0011 trial was conducted in patients who had

breast conservation with whole breast irradiation. The majority of breast cancers are located in the upper outer quadrant, and postlumpectomy radiation fields frequently will incidentally treat a portion of the lower axilla. The safety of omitting ALND for patients with involved SLNs who undergo mastectomy is not known. In these patients, the presence or absence of lymph node involvement and the number of positive axillary lymph nodes are important factors in determining the need for postmastectomy radiation therapy. Thus, many patients who undergo mastectomy still also will undergo ALND. Accurate intraoperative SLN assessment can avoid return to the operating room for completion lymph node dissection and may influence reconstructive decisions. Globally, patients with breast cancer undergo surgery in a very wide range of treatment settings. The role of intraoperative SLN pathologic assessment will vary significantly, depending on practice location and institutional pathology and surgical resources. There will be continued evolution of the role of completion dissection in the years to come as data from the ACO-SOG Z0011 trial mature. With the current clinical guidelines, the semiquantitative nature of the OSNA system provides a unique opportunity to tailor intraoperative surgical decision-making to the individual patient based on the relative lymph node tumor burden.

In summary, the current results demonstrated that the performance of the OSNA Breast Cancer System for detecting metastatic carcinoma in axillary SLNs in breast cancer was comparable to the performance of detailed histopathologic examination, and the results from this multi-center study support consideration of the OSNA system for evaluating SLNs in clinical practice. The assay does not require technologists who are skilled in molecular techniques or who have cytologic expertise, and it generates semiquantitative results, which makes it useful for both academic centers and community hospitals. This enables categorization of the metastasis beyond what is provided by qualitative results and makes the system particularly suitable for clinical decision-making. This molecular device may be useful both for intraoperative assessment of SLNs and for permanent, comprehensive SLN evaluation, because it provides rapid, standardized, and objective molecular testing of the lymph nodes.

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Table 1

Study Patient Demographics

Characteristic	No. of Patients (%)		
	SLN Negative[a]	SLN Positive[a]	All Patients
Patients	395 (79.6)	101 (20.4)	496 (100)
No. of SLNs (%)	906 (86.8)	138 (13.2)	1044 (100)
Age, y			
Mean [range]	59.2 [29-88]	56.9 [28-87]	58.8 [28-88]
<50	82 (20.8)	25 (24.8)	107 (21.6)
≥50	313 (79.2)	76 (75.3)	389 (78.4)
Sex			
Women	394 (99.8)	100 (99)	494 (99.6)
Men	1 (0.3)	1 (1)	2 (0.4)
Race			
Asian	9 (2.3)	4 (4)	13 (2.6)
Black	47 (11.9)	14 (13.9)	61 (12.3)
White	328 (83)	77 (76.2)	405 (81.7)
Others[b]	11 (2.8)	6 (5.9)	17 (3.4)
Type of surgery			
Breast-conserving surgery	302 (76.5)	60 (59.4)	362 (73)
Mastectomy	50 (12.7)	26 (25.7)	76(15.3)
Other	43 (10.9)	15 (14.9)	58 (11.7)
SLN mapping technique			
Blue dye	24 (6)	10 (9.9)	34 (6.9)
^{99m}Tc radiocolloid	89 (22.5)	18 (17.8)	107 (21.6)
Both	282 (71.4)	73 (72.3)	355 (71.6)
Diagnosis			
Invasive ductal	274 (69.4)	74 (73.3)	348 (70.2)
Invasive lobular	27 (6.8)	13 (12.9)	40 (8.1)
Invasive mixed	59 (14.9)	10 (10)	69 (13.9)
Other/unknown	21 (5.3)	3 (3)	25 (5.0)
No residual carcinoma	14 (3.5)	1 (1)	15 (3)
Tumor classification			
Tis	21 (5.3)	0 (0)	21 (4.2)
T1	282 (71.4)	45 (44.6)	327 (65.9)
T2	79 (20)	45 (44.6)	124 (25)
T3	1 (0.3)	4 (4)	5 (1)
Tx	12 (3.0)	7 (6.9)	19 (3.8)
Pathologic lymph node status[c]			
pN0	377 (95.4)	10 (9.9)	387 (78)
pN1	11 (2.8)	73 (72.3)	84 (16.9)
pN2	2 (0.5)	12 (11.9)	14 (2.8)

Characteristic	No. of Patients (%)		
	SLN Negative[a]	SLN Positive[a]	All Patients
pN3	1 (0.3)	3 (3)	4 (0.8)
pNx	4 (1.1)	3 (3)	7 (1.4)

SLN indicates sentinel lymph node; 99mTc, technetium 99m sulfur colloid; Tis, tumor in situ.

[a] Defined by the reference pathology laboratory.

[b] Also includes American Indians and Pacific Islanders.

[c] Lymph node status was determined by site pathology.

Table 2

Results of Reference Pathology and the One Step Nucleic Acid Amplification Breast Cancer System

	Histopathology[a]				
OSNA[b]	**Macro**	**Micro**	**ITC**	**Negative**	**Total**
++	77	9	1	8	95
+	9	12	0	29	50
−	9	22	14	854	899
Total	95	43	15	891	1044

OSNA indicates the One Step Nucleic Acid Amplification Breast Cancer System; Macro, macrometastasis; Micro, micrometastasis; ITC, isolated tumor cells.

[a] Alternate 1-mm slices of sentinel lymph nodes were analyzed by either reference pathology in a central pathology laboratory or by using the OSNA Breast Cancer System. Criteria for macrometastasis, micrometastasis, ITCs, or negative findings followed *American Joint Committee on Cancer Staging Manual* (6th edition) guidelines for histopathology.

[b] OSNA results were classified as ++ (macrometastasis), + (micrometastasis), or − (negative), as determined by a pre-established cutoff number of cytokeratin 19 messenger RNA copies.

Table 3

Overall Performance of the One Step Nucleic Acid Amplification Breast Cancer System Compared With Reference Pathology

Variable	No. of SLNs	Performance % (95% CI)				
		Sensitivity	Specificity	Agreement	NPV	PPV
OSNA Results						
Across cohort						
Before DCA	1044	77.5 (69.7-84.2)	95.8 (94.3-97.0)	93.4 (91.7-94.8)	96.6 (95.1-97.6)	73.8 (65.8-80.7)
After DCA	1018	82.7	97.7	95.8	NA	
Macrometastasis before DCA	1044	81.1 (71.7-88.4)	98.1 (97.0-98.9)	96.6 (95.3-97.6)	98.1 (97.0-98.9)	81.1 (71.7-88.4)
Pure lobular carcinoma	84	88.2 (63.6-98.5)	98.5 (92.0-100.0)	96.4 (89.9-99.3)	97.1 (89.8-99.6)	93.8 (69.8-99.8)

CI indicates confidence interval; SLNs, sentinel lymph nodes; DCA, discordant case analysis; OSNA, the One Step Nucleic Acid Amplification Breast Cancer System.

Table 4

Results of Discordant Case Analysis

Discordant Samples (n = 71)	SLN Count
Resolved[a]	28
Tissue allocation bias	19
Site sample mix-up	6
Change in pathology result	3
Unresolved	43

SLN indicates sentinel lymph node.

[a] For the 1044 comparisons between reference pathology and the One Step Nucleic Acid Amplification Breast Cancer System, there were 71 discordant results. Follow-up pathologic and molecular investigations allowed for grouping of discordant samples into the 3 categories shown.

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Table 5

Detailed Analysis of Discordant Samples[a]

		Western Blot Analysis		qRT-PCR						
		CK19		CK19		SPDEF		FoxA1		
Lymph Node ID	OSNA Results[b]	Concentration, ng/μL	Result	Ct	Result	Ct	Result	Ct	Result	Final Disposition
Reference pathology results: Negative or ITC										
06-043-02	++	NA[c]								Concordant
05-067-01	++	0.47	+	28.9	+	29.1	+	36.7	−	Concordant
05-050-01	++	16.19	+	26.4	+	27.9	+	34.4	−	Concordant
04-012-02	++	3.39	+	25.9	+	28.3	+	31.8	+	Concordant
03-027-02	++	0.27	+	29.4	+	31.4	+	36.8	−	Concordant
03-003-01[d]	++	NA[e]								Concordant
02-033-02[d]	++	0.23	+	28.9	+	29.9	+	34.1	−	Concordant
02-001-02	++	0.09	−	28.6	+	30.6	+	32.5	+	Concordant
04-007-03[d]	++	1.17	+	26.7	+	28.9	+	30.6	+	Concordant
Reference pathology results: Macrometastasis										
09-088-01	−	0.21	+	26.6	+	30.2	+	34.8	−	Discordant
08-037-01	−	0.22	+	29.3	+	31.6	−	35.6	−	Discordant
03-027-03	−	5.32	+	21.9	+	24.7	+	29.8	+	Discordant
02-054-02	−	3.79	+	21.6	+	23.5	+	26.8	+	Discordant
02-054-01	−	0.99	+	22.4	+	24.1	+	28.7	+	Discordant
02-052-01	−	7.64	+	21.9	+	24.4	+	31.0	+	Discordant
02-029-03	−	0.31	+	23.9	+	20.5	+	25.3	+	Discordant
01-070-01	−	0.29	+	28.3	+	29.7	+	36.2	−	Discordant
08-048-01	−	0.07	−	34.2	−	35.1	−	36.4	−	Concordant

qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; CK19, cytokeratin 19; SPDEF, SAM-pointed domain-containing ets transcription factor; FoxA1, forkhead box A1; ID, identification; OSNA, the One Step Nucleic Acid Amplification Breast Cancer System; ITC, isolated tumor cells; Ct, cycle time; NA, not available; DCA, discordant case analysis.

[a]Molecular assessments included determining CK19 protein levels by Western blot analysis (cutoff level for positive detection, 0.13 ng/μL) and messenger RNA levels for CK19 (cutoff ct, 31.5), SPDEF (cutoff, 31.6), and FOXA1 (cutoff, 33.8) by qRT-PCR.

[b]OSNA results were classified as ++ (macrometastasis) or − (negative).

[c] The reference pathology laboratory revised the results to positive (micrometastasis 0.7 mm) at DCA.

[d] These were mislabeled samples that were retested and identified as concordant.

[e] This sample was mislabeled and retested as negative by OSNA.

Table 6

Results of Imprint Cytology and the One Step Nucleic Acid Amplification Breast Cancer System Compared With Reference Pathology[a]

	Reference Pathology				
	Macro	**Micro**	**ITC**	**Negative**	**Total**
Imprint cytology, n =532[b]					
Positive	41	10	0	3	54
Negative	14	15	8	436	473
Indeterminate	1	0	0	4	5
Total	56	25	8	443	532
OSNA, n =532[c]					
++	48	6	1	4	59
+	4	7	0	14	25
−	4	12	7	425	448
Total	56	25	8	443	532

Macro indicates macrometastasis; Micro, micrometastasis; ITC, isolated tumor cells; OSNA, the One Step Nucleic Acid Amplification Breast Cancer System.

[a] In total, 532 sentinel lymph nodes that were obtained from 272 patients enrolled from 7 clinical sites were evaluated intraoperatively by imprint cytology before either OSNA or postoperative reference pathology. Criteria for macrometastasis, micrometastasis, ITCs, or negative findings followed *American Joint Committee on Cancer Staging Manual* (6th edition) guidelines.

[b] Imprint cytology results were classified as positive, negative, or indeterminate.

[c] OSNA results were classified as ++ (macrometastasis), + (micrometastasis), or − (negative), as determined by a pre-established cutoff number of cytokeratin 19 messenger RNA copies.

Table 7

Performance Values for the One Step Nucleic Acid Amplification Breast Cancer System and Imprint Cytology[a]

	Performance Value % (95% CI)				
	Before DCA			After DCA	
Variable	**OSNA**	**Imprint Cytology**	***P***	**OSNA**	***P***
Agreement	93.4 (91.0-95.4)	93.8 (91.4-95.7)	NS	95.8 (93.7-97.3)	NS
Sensitivity	80.2 (69.9-88.3)	63.0 (51.5-73.4)	.0229	85.5 (75.6-92.6)	.0018
Specificity	95.8 (93.5-97.4)	99.3 (98.1-99.9)	.0007	97.5 (95.6-98.8)	.0324
PPV	77.4 (67.0-85.8)	94.4 (84.6-98.8)	.0082	85.5 (75.6-92.6)	.1519
NPV	96.4 (94.3-97.9)	93.7 (91.2-95.7)	.0691	97.5 (95.6-98.8)	.0061

CI indicates confidence interval; DCA, discordant case analyses; OSNA, the One Step Nucleic Acid Amplification Breast Cancer System; NS, nonsignificant; PPV, positive predictive value; NPV, negative predictive value.

[a]Agreement with reference pathology, assay sensitivity, specificity, PPV, and NPV, together with 95% CIs, were calculated both for the OSNA Breast Cancer System and imprint cytology and were compared with reference pathology. Values are before and after DCA.

Table 8

Results for Lobular Carcinoma

	Histopathology[a]				
OSNA[b]	**Macro**	**Micro**	**ITC**	**Negative**	**Total**
++	10	3	0	1	14
+	1	1	0	0	2
−	0	2	4	62	68
Total	11	6	4	63	84

OSNA indicates the One Step Nucleic Acid Amplification Breast Cancer System; Macro, macrometastasis; Micro, micrometastasis; ITC, isolated tumor cells.

[a] Lobular carcinoma deposits in sentinel lymph nodes were analyzed either by reference pathology in a central pathology laboratory or by using the OSNA Breast Cancer System. Criteria for macrometastasis, micrometastasis, ITCs, or negative findings followed *American Joint Committee on Cancer Staging Manual* (6th edition) guidelines.

[b] OSNA results were classified as ++ (macrometastasis), + (micrometastasis), or − (negative), as determined by a pre-established cutoff number of cytokeratin 19 messenger RNA copies.