Comparative Study of the One-step Nucleic Acid Amplification Assay and Conventional Histological Examination for the Detection of Breast Cancer Sentinel Lymph Node Metastases

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Intraoperative sentinel lymph node (SLN) biopsy is widely used in patients with early-stage breast cancer and is conventionally performed using hematoxylin and eosin-based histological examination. The one-step nucleic acid amplification (OSNA) assay is a molecular diagnostic tool and a semi-automated lymph node examination method. The purpose of this study was to compare the performance of the OSNA assay and conventional histological examination with frozen sections (FSs) by using 111 SLN biopsy samples from 89 patients at the Tokai University Hospital. The SLN samples were split into 3 slices: the middle slice was used for FS histological examination and the other slices were used for the OSNA assay. The McNemar test was used to compare the differences in the sensitivity and specificity between the OSNA assay and FS histological examination. The sensitivity of the OSNA assay (97.1%) was less than that of FS histological examination (100%), but this difference was not statistically significant ($P = 0.125$). The specificity of both the methods was identical (96.9%). Despite previously published results suggesting that the OSNA assay is as reliable as histological examinations, our results indicate that this assay often fails to detect micrometastases or isolated tumor cells in SLNs.

Key words: breast cancer, sentinel lymph node, CK19, OSNA assay

INTRODUCTION

Intraoperative sentinel lymph node (SLN) biopsy is widely used for patients with early-stage breast cancer, who are clinically negative for lymph node metastases. The SLN status is a highly accurate predictor of overall axillary lymph node status, and patient morbidity is known to decrease on omitting unnecessary axillary lymph node dissection (ALND) when the SLN is negative for metastasis [1, 2]. However, detailed examinations result in a heavy workload for pathologists. Conventional procedures for intraoperative assessment of SLNs are hematoxylin and eosin (H&E)-based histological examination of frozen sections (FSs) or cytological observation of touch-imprints [3]. These procedures require experienced pathologists and are associated with numerous technical difficulties including complicated specimen preparation, variability in the quality of the prepared specimen, and an inconsistent diagnostic accuracy. Further, the observation is limited to only a part of the lymph node.

Recently, several molecular detection procedures for lymph node metastases have been developed, and they represent the most promising solution to this problem [4–7]. The one-step nucleic acid amplification (OSNA) assay is a rapid molecular diagnostic device and a semi-automated lymph node examination method that uses molecular biological techniques to amplify cytokeratin 19 (CK19) mRNA from the lymph node [8]. In the normal mammary gland, luminal cells mainly express CK7, CK8, CK18, and CK19, whereas basal/myoepithelial cells express CK5/6, CK14, and CK17. The expression of the CK19 in breast adenocarcinoma is reported to be as high as 98.4%[9]. Quantitative reverse transcription polymerase chain reaction for CK19 and mammaglobin 1 was reported to be almost as accurate (94.1% sensitivity and 98.6% specificity) as conventional histological examination for detecting SLN metastases [10].

The OSNA assay has a number of advantages over the conventional step-sectioning FS histological examination, including the possibility of examining the whole lymph node by using a standardized procedure with less inter-observer variability. Data from a number of recent studies have revealed that the OSNA assay is as accurate as conventional histological examination for the detection of SLN metastases [7, 8, 11, 12] and is therefore an effective alternative tool for their intraoperative detection. Nevertheless, CK19 is the only marker used in the OSNA assay, which might give rise to false-negative results due to low CK19 expression in some cancer cells and false-positive results due

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to the presence of mammary luminal cells [13]. The purpose of this study was to compare the sensitivity and specificity of the OSNA assay and FS histological examination for the intraoperative detection of SLN metastases by using postoperative formalin-fixed tissue histological diagnoses as reference.

MATERIALS AND METHODS

Patients

A total of 89 patients with early-stage breast cancer without clinical signs of lymph node metastasis, and who underwent an SLN biopsy between March 2009 and March 2010 at the Tokai University Hospital, Kanagawa, Japan, were included in this study. This study was approved by the ethics committee of our institution, and each patient provided informed consent.

Identification of SLNs

SLNs were identified via the combined use of the radioisotope (RI) method and the dye staining method. The RI tracer technetium (99mTc)-phytate was injected into the dermis of the areola on the morning of the surgery. In addition, 4 mL of a vital dye, indigo carmine, was injected into the dermis of the areola at the time of surgery. Before resection of the primary tumor, SLNs were identified by using a handheld gamma-probe (Navigator GPS; Tyco Healthcare Japan; Tokyo, Japan) and vessel and lymph node staining.

Sampling methods

SLNs were defatted after sentinel lymph node biopsy (SLNB), and were split into 3 slices using a 2-mm tissue cutter. The middle slice was used for the FS histological examination and postoperative formalin-fixed tissue histological examination. The other slices were used as the specimen for the OSNA assay. ALND was only performed if lymph node metastasis was detected on FS histological examination.

FS histological examination

The middle slice of the resected SLN was examined via FS histological examination with H&E staining. The remaining portion of this slice was preserved as a final histological section.

OSNA assay

All OSNA assays (Sysmex, Kobe, Japan) were carried out according to the manufacturer's instructions. Three different calibrators with a known CK19 mRNA copy number were used to establish a standard curve on an RD-100i instrument (Sysmex). Node tissues were homogenized in 4 mL of the LYNORHAG homogenizing buffer (Sysmex). The homogenate was briefly centrifuged and used as a template for amplification. Amplification of CK19 mRNA was performed on the automated RD-100i instrument with the ready-to-use reagent LYNOAMP (Sysmex) that is provided as part of the detection kit, which also contains a primer-nucleotide mix, enzymes, CK19 mRNA calibrators, homogenate, diluted homogenate, and positive and negative controls. All results were presented on the RD-100i instrument as qualitative categories (++, +, I, −) according to the CK19 mRNA copy number/L: (−), <250 copies in the homogenate only; (I), <250 copies in the homogenate and >250 copies in the diluted homogenate; (+), 250−5000 copies in the homogenate; and (++), >5000 copies in the homogenate. The ++, +, and I categories were considered positive result [8].

Postoperative formalin-fixed tissue histology

All node blocks used for histological evaluation were fixed in 10% formalin, embedded in paraffin, and stained by using H&E. Metastases > 0.2 mm were considered positive in this study, and were classified according to the sixth edition of the American Joint Committee on Cancer staging system. The presence of macrometastases (>2 mm) or micrometastases (>0.2−2 mm, pT1mi) were considered to define a positive node, while the presence of isolated tumor cells (ITCs) (≤0.2 mm, pT0[i+]) did not constitute a positive node.

Immunohistochemical staining for CK19 by using the RCK108 monoclonal antibody (Dako) was also performed for cases that showed negative results on the OSNA assay and positive results on FS histological examination (i.e., OSNA negative/FS positive cases).

Statistical methods

Sensitivity and specificity were determined on the basis of the number of lymph nodes, not the number of patients. The McNemar test was used to test differences in the accuracy (sensitivity and specificity) between the findings obtained with the OSNA assay and FS histological examination. A p-value of <0.05 was considered statistically significant. The analyses were performed by using SPSS version 21 (SPSS Inc., Chicago, IL).

RESULTS

A total of 111 SLNs were surgically resected from 89 patients with early-stage breast cancer who did not show any clinical signs of lymph node metastasis; the average number of SLNs was 1.25 per patient. The detection rate of SLNs was 100% (89/89) by using the RI method or the dye method, or both. Patient characteristics are summarized in Table 1. In this study, the mean patient age at the time of surgery was 56 years (range, 29−81 years), and most patients had tumor stages of <T3 (92%). Fifty-seven patients had invasive ductal carcinoma, 4 had invasive lobular carcinoma, 20 had ductal carcinoma in situ, and 7 had others types of breast cancer. Seventy-four (83.1%) patients underwent breast-conserving surgery, 15 (16.8%) patients underwent mastectomy, 72 (80.9%) patients underwent SLNB alone following a negative SLN finding, and in 17 (19.1%) patients, ALND was converted to SLNB following a positive SLN finding.

On the basis of the results of the OSNA assay, 13 of the 111 SLNs (11.7%) were positive for metastasis and 98 (88.3%) were negative. In contrast, 16 SLNs (14.4%) were positive according to the results of FS histological examination, and 95 (85.6%) were negative (Table 2). Six nodes were OSNA negative/FS positive, and 3 were OSNA positive/FS negative. In all of these 9 discordant cases, the results of FS histological examination agreed with those of the final histology. The 6 OSNA negative/FS positive cases were subjected to further analysis. Five (83.3%) of these nodes stained weakly for CK19, the other stained strongly. Two (33.3%) of the

Table 1 Characteristics of patients with breast cancer enrolled in this study

Mean age, years (range)	56 (29–81)
Tumor stage	
Tis	21
T1	21
T2	40
T3	7
Postoperative final diagnosis	
Invasive ductal carcinoma	57
Invasive lobular carcinoma	4
Ductal carcinoma in situ	20
Other cancer type	7
Not known	1
ER status	
Positive	21
Negative	63
Not known	5
HER-2 status	
Positive	71
Negative	12
Not known	6
Breast surgery	
Breast conserving surgery	74
Mastectomy	15
Axillary surgery	
SLNB	72
ALND (following positive SLN)	17

Abbreviations: ALND, axillary lymph node dissection; ER, estrogen receptor; HER-2, human epidermal growth factor receptor; SLN, sentinel lymph node; SLNB, sentinel lymph node biopsy; Tis, ductal carcinoma in situ

nodes show stained only in intraductal. Two (33.3%) of the nodes showed heterogeneous CK19 staining, and the other 4 (66.7%) nodes exhibited homogenous staining. On the basis of formalin-fixed tissue histological examination, 3 (50%) nodes were found to contain ITCs, 1 (16.7%) node contained a micrometastasis, and 2 (33.3%) nodes contained macrometastases (Table 3).

The accuracy of the OSNA assay compared to formalin-fixed tissue histological examination was 93.7% (104/111), with a sensitivity of 71.4% (10/14), a specificity of 96.9% (94/97), a positive predictive value (PPV) of 76.9% (10/13), and a negative predictive value (NPV) of 95.9% (94/98) (Table 4). The accuracy of the FS histological examination compared to formalin-fixed tissue histological examination was 97.3% (108/111), with a sensitivity of 100% (14/14), a specificity of 96.9% (94/97), a PPV of 82.4% (14/17), and a NPV of 100% (94/94) (Table 5).

Three nodes were positive on FS histological examination but negative on formalin-fixed tissue histological examination, with metastases of 50 μm, 125 μm, and 100 μm; all were considered to have ITCs and were thus pathologically negative for metastasis.

The sensitivity of the OSNA assay was less than that of FS histological examination, but this difference was not statistically significant (71.4% vs. 100%; $P = 0.125$); however, both methods had equal specificity (96.9%) (Table 6).

DISCUSSION

In this study, we found that the sensitivity of the OSNA assay for detecting metastases in SLNs was 71.4%, which is lower than that reported from previous studies [4, 7, 11, 12, 14], although the specificity, PPV, and NPV were similar [4, 7, 11, 12, 14]. When compared to the definitive results of postoperative formalin-fixed tissue histological examination, the accuracy, sensitivity, and specificity of FS histological examination were 97.3%, 100%, and 96.9%, respectively. It is well established that conventional intraoperative histological examination during SLNB yields a high (10–30%) false-negative rate for metastatic foci because only a few thin sections from a lymph node are examined in this technique [15]. However, in this study, conventional FS histological examination accurately detected SLN metastases. There have been 14 previous studies comparing the accuracy of the OSNA assay to pathology-based methods for the detection of lymph node metastases, in which the reported sensitivity and specificity of the OSNA assay were 75.0–98.1% and 91.7–98.4%, respectively; the OSNA assay was judged to be statistically as reliable as the pathological examination [7, 11, 13, 14, 16–22].

We found 6 nodes that were OSNA negative/FS positive, among which 5 (83.3%) stained only weakly for CK19, and the false-negative result was thus due to low CK19 expression. The incidence of low CK19 protein expression in breast cancer has been reported to be 0–20.5% [9, 23, 24], although the actual incidence of tumors with low CK19 expression remains unclear. In this study, the incidence of discordance between the OSNA assay and the FS histological examination caused by low expression of CK19 was 5.6%.

Three (50%) of the 6 OSNA negative/FS positive nodes contained ITCs, and 1 (16.7%) node was found to contain a micrometastasis on formalin-fixed tissue histological examination. In addition, 2 (33.3%) of these nodes showed heterogeneity for CK19 staining. A small metastasis is more likely to be confined to a single section; therefore, it might only be detected with either of the tests. However, it is exceedingly difficult to evaluate the actual sensitivity of the OSNA assay because there is no SLN tissue remaining for histological evaluation after RNA extraction from the section of the SLN. Osako *et al.* [4] reported that more micrometastases could be detected with the OSNA assay than with the conventional pathological method, although ITCs and micrometastases may have been recorded as false-negative results. The clinical significance of micrometastases and ITCs in the node was ambiguous, and a precise, initial evaluation of SLN metastasis is important for the accurate assessment of clinical stage and the appropriate selection of adjuvant treatment for each patient. Further studies are necessary to better understand the significance of positive OSNA assay results for SLN ITCs or micrometastases.

A particular advantage of the OSNA assay over pathology is that the former allows the semi-quantitative

Table 2 Comparison of results between the OSNA assay and FS histological examination

89 patients 111 SLNs		FS histology		
		Positive	Negative	Total
OSNA	Positive*	10	3	13
	Negative †	6	92	98
	Total	16	95	111

Abbreviations: OSNA, one-step nucleic acid amplification; FS, frozen section; SLN, sentinel lymph node
NOTE: Rate of accordance: 91.0%
*CK19 mRNA ≥ 2.5×10^2 copies/μL
†CK19 mRNA < 2.5×10^2 copies/μL

Table 3 Further analysis of lymph nodes in discordant cases (OSNA negative/FS positive)

	Intraoperative		Immunohistochemistry for CK19			Postoperative histology
	OSNA	FS	Proportion (%)	Intensity	Heterogenity	Diameter of metastatic lesion
1	–	+	>90%	strong	–	1500μm (Mi)
2	–	+	40%	weak	+	50μm (ITC)
3	–	+	10%	weak	–	125μm (ITC)
4	–	+	<5%	weak	–	100μm (ITC)
5	–	+	Intraductal* 70%	weak	+	2000μm (Ma)
6	–	+	intraductal* 80%	weak	–	2500μm (Ma)

Abbreviations: Ma, macrometastasis; Mi, micrometastasis; ITC, isolated tumor cell; OSNA, one-step nucleic acid amplification; FS, frozen section
*: Proportion of Immunohistochemistry for CK19 in intraductal carcinoma cell

evaluation of total tumor volume in the node. All the results of this assay are presented as qualitative categories (++, +, I, −). In this study, the ++, +, and I categories were considered positive, and the significance of individual categories was therefore not investigated. Tsujimoto *et al* [8]. studied the possible significance of these individual categories, and found that the + and ++ categories were associated with the presence of a micrometastasis and macrometastasis, respectively. Further studies are needed to evaluate the significance of individual categories determined with the OSNA assay.

This study had a number of limitations, including a selection bias in the study design. The surgically excised SLNs were split into 3 slices by using a 2-mm tissue cutter; the middle slice was used for FS histological examination and postoperative formalin-fixed tissue histological examination, and the other slices were used for the OSNA assay. The amount of specimen used for the OSNA assay may thus have been insufficient in some cases.

CONCLUSIONS

Our findings suggested that the sensitivity of the OSNA assay for detecting breast cancer metastases in axillary SLNs was inferior to that of conventional histological examination (FS), which had a very high sensitivity, although the results support the use of the OSNA assay for evaluating SLNs in clinical practice. Many previous studies have shown that the OSNA assay is as reliable as pathology-based methods for metastases detection and have indicated that it could be an alternative method for the examination of SLNs. However, our findings indicate that the OSNA assay may fail to detect SLNs with micrometastases or ITCs. A larger study may allow the development of a more standardized approach for SLN evaluation with the OSNA assay.

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Table 4 Comparison of the OSNA assay with final postoperative histological examination

89 patients 111 SLNs		Final histology		
		Positive	Negative	Total
OSNA	Positive*	10	3	13
	Negative †	4	94	98
	Total	14	97	111

Abbreviations: OSNA, one-step nucleic acid amplification; SLN, sentinel lymph node
*CK19 mRNA ≥ 2.5×10^2 copies/μL
†CK19 mRNA < 2.5×10^2 copies/μL

Table 5 Comparison of the results with FS histological examination and the final postoperative histological examination

89 patients 111 SLNs		Final histology		
		Positive	Negative	Total
FS histological examination	Positive	14	3	17
	Negative	0	94	94
	Total	14	97	111

Abbreviations: FS, frozen section; SLN, sentinel lymph node

Table 6 Performance of the OSNA assay and FS histological examination in assessing breast sentinel lymph nodes

	Sensitivity (%)	Specificity(%)	PPV (%)	NPV (%)
OSNA assay	71.4	96.9	76.9	95.9
FS histological examination	100	96.9	82.4	100

Abbreviations: OSNA, one-step nucleic acid amplification; FS, frozen section; NPV,negative predictive value; PPV, positive predictive value

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