Whole sentinel lymph node analysis by a molecular assay predicts axillary node status in breast cancer

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BACKGROUND: The one-step nucleic acid amplification (OSNA) assay is a rapid procedure for the detection of lymph node (LN) metastases using molecular biological techniques. The aim of this study was to assess the reliability of the whole sentinel lymph node (SLN) analysis by the OSNA assay as a predictor of non-SLN metastases.

METHODS: Consecutive 742 patients with breast cancer were enroled in the study. The association of non-SLN or $\geqslant 4$ LN metastases with clinicopathological variables was investigated using multivariate logistic analysis.

RESULTS: In total, 130 patients with a positive SLN who underwent complete axillary LN dissection were investigated. The frequency of non-SLN metastases in patients who were OSNA+ and ++ was 19.3% and 53.4%, respectively, and that in patients with $\geqslant 4$ LN metastases who were OSNA+ and ++ was 7.0% and 27.4%, respectively. The cytokeratin 19 (CK19) mRNA copy number ($\geqslant 5.0 \times 10^3$; OSNA++) in the SLN was the most significant predictors of non-SLN metastases ($P = 0.003$). The CK19 mRNA copy number ($\geqslant 1.0 \times 10^5$) in the SLN was the only independent predictor of $\geqslant 4$ LN metastases ($P = 0.014$).

CONCLUSION: Whole SLN analysis using the OSNA assay could become a valuable method for predicting non-SLN and $\geqslant 4$ LN metastases.

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Intraoperative sentinel lymph node (SLN) biopsy is widely applied to patients with early-stage breast cancer, who are clinically negative for lymph node (LN) metastases. Whether the SLN is involved is a highly accurate predictor of overall axillary LN status, and the patient morbidity rate has been reduced by omitting unnecessary axillary lymph node dissection (ALND) when the SLN is negative for metastases (Veronesi *et al*, 1997; Krag *et al*, 2010). Although ALND remains a standard surgical procedure for SLN-positive patients because of its potential prognostic and therapeutic benefit (Lyman *et al*, 2005), no additional involved axillary LNs are found after complete ALND in almost half of the patients with positive SLNs (Chu *et al*, 1999; Reynolds *et al*, 1999). Thus, it has been suggested that ALND may be avoided in certain patients, including those with a positive SLN. Recently, it was reported that non-SLN involvement negatively influenced patient outcome, regardless of the number of positive LNs (Jakub *et al*, 2011). Many models predicting non-SLN involvement in SLN-positive breast cancers have been reported (Van Zee *et al*, 2003; Degnim *et al*, 2005; Pal *et al*, 2008). However, conventional histological examination of SLNs are subject to interobserver variability and are limited in their ability to detect metastases accurately, because only a portion of the LN tissue is used in the preparation of histological sections. In contrast, a molecular technique that can evaluate the entire LN tissue using a standardised procedure would have less interobserver variability. The one-step nucleic acid amplification (OSNA) assay (Sysmex, Kobe, Japan) is a rapid molecular diagnostic device and a semi-automated LN examination method that uses molecular biological techniques to amplify cytokeratin 19 (CK19) mRNA from the LN (Tsujimoto *et al*, 2007). Accurate intraoperative detection of SLN metastases and prediction of non-SLN metastases may be helpful for ALND decision making. Recent studies revealed that the OSNA assay was as accurate as conventional histological examinations for the detection of SLN metastases (Tsujimoto *et al*, 2007; Tamaki *et al*, 2009; Snook *et al*, 2011). However, few reports have evaluated whole SLN tissue using the OSNA assay to eliminate tissue allocation bias (Osako *et al*, 2011; Sagara *et al*, 2011; Castellano *et al*, 2012; Godey *et al*, 2012). To the best of our knowledge, this study is the first to demonstrate that the CK19 mRNA copy number in whole SLN analysis using the OSNA assay is the most important predictive factor of non-SLN metastases, and that a higher copy number of CK19 mRNA is significantly associated with four or more axillary LN metastases.

MATERIALS AND METHODS

Patients

A total of 763 consecutive patients with clinical and physical LN-negative invasive breast cancer, who underwent an SLN biopsy between August 2009 and August 2011 at the Sagara Hospital, Kagoshima, Japan, were used in this study. The SLNs of the patients were assayed by the OSNA assay for SLN metastasis detection. Noninvasive breast carcinoma cases and those who underwent neoadjuvant therapy were excluded from the study. The SLNs were identified in 752 of the 763 patients (98.6%). Ten cases with apparent macrometastases were excluded from this study, because the nodal tissues were processed for frozen section diagnosis. Finally, 742 cases were enroled in this study. Clinicopathological data, including age, clinical tumour size, pathological tumour size, histological type, nuclear grade, presence of lymphovascular invasions (LVIs), oestrogen receptor and HER2 status and type of breast cancer surgery were retrospectively collected. The staging of the cases was classified according to the TNM AJCC 7th edition. The patient's characteristics are shown in Table 1.

Detection of the SLN

First, 0.5 ml of technetium-99m phytate (18.5 MBq, FUJIFILM RI PHARMACY, Tokyo, Japan) mixed with 0.5 ml of 1% lidocaine hydrochloride was injected into the dermis of the areola 4–7 h before surgery. All patients underwent preoperative static scintigraphic imaging in anterior and oblique projections using a dual-head gamma camera with a low-energy, high-resolution collimator (4-min acquisition in a 256×256 matrix) 30 min to 1 h after the injection of the radio tracer. The locations of the axillary and non-axillary SLNs were marked on the skin. After general anaesthesia, 2 ml of Patent Blue V dye (Laboratoire Guerbet, Aulnay-sous-Bois, France) was diluted to 5 ml with saline and injected into the dermis of the areola immediately before the first incision was made. The SLNs were identified by blue dye mapping and handheld gamma probe detection (Navigator GPS, Radiation Monitoring Device Instruments, Watertown, MA, USA) during operation. All LN that stained blue or those with radioactive counts 50 times higher than the background count were defined as SLNs.

OSNA assay

After the fatty tissue was removed, the SLN was weighed and cut along the short axis, and whole SLN tissues were processed for the OSNA assay.

The OSNA assay, which is based on the principles of the reverse transcription loop-mediated isothermal amplification method, has been processed as previously described (Tsujimoto *et al*, 2007). The LN was assessed as OSNA– when the CK19 mRNA copy number was fewer than 2.5×10^2 copies μl^{-1}, OSNA+ when it was between 2.5×10^2 and 5.0×10^3 copies μl^{-1}, and OSNA++ when it was more than 5.0×10^3 copies μl^{-1}. The OSNA assay is sometimes inhibited by inhibitory materials (Osako *et al*, 2011; Castellano *et al*, 2012), resulting in false-negative (<250 copies μl^{-1}) reactions that may be resolved as positive ($\geqslant 250$ copies μl^{-1}) reactions by simple dilution (1:10). However, the values of these reactions after dilution are less reliable for the quantitative assessment and were evaluated as +inhibition (+I).

Ethical considerations

This study was approved by the Ethics Committee in the Social Medical Corporation Hakuaikai. We obtained informed consent from all patients who participated in this study.

Table I Patient characteristics

Characteristics	No.	%
Total number of patients	742	100.0
Age (years)		
<50	217	29.2
⩾50	525	70.8
Tumour size		
Tis	29	3.9
T1	369	49.7
T2	318	42.9
T3	26	3.5
Pathological T classification		
pT1	528	71.2
pT2	201	27.1
pT3	13	1.8
Nuclear grade		
1	404	54.4
2	202	27.2
3	136	18.3
Histological type		
Invasive ductal carcinoma	659	88.8
Invasive lobular carcinoma	41	5.5
Others	42	5.7
Oestrogen receptor status		
Positive	614	82.7
Negative	126	17.0
Unknown	2	0.3
HER2 status		
Positive	105	14.2
Negative	622	83.8
Unknown	15	2.0
Lymphovascular invasion		
Absent	576	77.6
Present	166	22.4
Type of breast surgery		
Conservative	630	84.9
Mastectomy	112	15.1
No. of removed sentinel nodes		
1	601	81.0
2	130	17.5
3	10	1.3
4	1	0.1

Statistical analyses

Statistical analyses were performed using SPSS (SPSS, Chicago, IL, USA). Associations between the different parameters were assessed using the χ^2-test. A difference was considered significant if the *P*-value was <0.05. Factors were evaluated in a multivariate logistic regression model to identify independent factors associated with the presence of non-SLN metastases and four or more LN metastases. For each factor, the likelihood of positive non-SLNs and four or more LN metastases were estimated by the odds ratio and the 95% confidence interval (CI).

RESULTS

Clinicopathological characteristics

The SLN metastases were detected in 148 out of 742 patients (19.9%). Of the 148 patients, 66 (44.6%), 73 (49.3%) and 9 (6.1%) were measured as OSNA+, ++ and +I, respectively. Nine

OSNA +I patients were excluded, owing to the presence of inhibiting materials, which make the assay less reliable. Of these SLN-positive patients, 130 underwent immediate ALND. Thus, a total of 130 patients (i.e., 57 OSNA+ and 73 OSNA++) were found to be eligible for our study. The median age was 54 years (range: 31–82). The mean number of SLNs per patient was 1.3 (range: 1–4), and all SLNs were located at level I. The mean number of dissected LNs per patient was 12.8 (range: 4–35).

Association of non-SLN metastases and four or more LNs metastases with clinicopathological parameters

The frequency of non-SLN metastases in the OSNA+ and OSNA++ groups was 19.3% (11 out of 57) and 53.4% (39 out of 73), respectively. The frequency of four or more LN metastases in the OSNA+ and OSNA++ groups was 7.0% (4 of 57) and 27.4% (20 of 73), respectively. In patients possessing a CK19 mRNA copy number of $\geqslant 1.0 \times 10^5$ copy number of CK19 mRNA, the frequency of four or more LN metastases was 35.3% (12 out of 34). The CK19 mRNA copy number was significantly correlated with non-SLN ($P < 0.001$) and four or more LN metastases ($P = 0.003$) (Table 2). In multivariate logistic regression analysis, the pathological tumour size ($P = 0.024$), LVI ($P = 0.019$) and $\geqslant 5.0 \times 10^3$ CK19 mRNA copy number in the SLN ($P = 0.003$) were identified as significant predictive factors of non-SLN metastases (Table 3). A higher CK19 mRNA copy number ($\geqslant 1.0 \times 10^5$) in the SLN was identified as a significant predictive factor for four or more LN metastases ($P = 0.014$; Table 4).

DISCUSSION

The need for complete ALND in patients diagnosed as SLN-positive has been questioned. Approximately 40–60% of patients with positive SLNs have been found to have no additional non-SLN metastases after complete ALND (Chu et al, 1999; Reynolds et al, 1999). These patients might therefore receive no therapeutic benefit from complete ALND. The updated guidelines of the National Comprehensive Cancer Network suggest the omission of ALND, even in cases with SLN metastases, when the cases meet all of the following criteria: T1 or T2 tumour, 1 or 2 positive SLNs, breast conserving therapy, whole breast radiotherapy planned and no neoadjuvant chemotherapy (NCCN, 2012). Because of the controversial prognostic and therapeutic benefits of ALND and concerns regarding its potential complications, many surgeons do not perform complete ALND in a portion of SLN-positive patients.

It has been reported that non-SLN involvement negatively influences patient outcome irrespective of the number of positive LNs (Jakub et al, 2011). Many factors, such as tumour size, the

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Table 2 Association between the axillay nodal status and clinicopathological findings in SLN-positive patients

	Total number (n = 130)	Non-SLN metastases			≥4 Node metastases		
		Present (n = 50)	Absent (n = 80)	P-value	Present (n = 24)	Absent (n = 106)	P-value
Age (years)							
<50	41	15 (36.6)	26 (63.4)	0.765	8 (19.5)	33 (80.5)	0.834
≥50	89	35 (39.3)	54 (60.7)		16 (18.0)	73 (82.0)	
Tumour size (cm)							
≤2	54	14 (25.9)	40 (74.1)	0.013	6 (11.1)	48 (88.9)	0.069
>2	76	36 (47.4)	40 (52.6)		18 (23.7)	58 (76.3)	
Histological type							
Invasive ductal carcinoma + others	127	49 (38.6)	78 (61.4)	0.853	24 (18.9)	103 (81.1)	0.404
Invasive lobular carcinoma	3	1 (33.3)	2 (66.7)		0 (0.0)	3 (100.0)	
Pathological tumour size (cm)							
≤2	70	18 (25.7)	52 (74.3)	0.001	8 (11.4)	62 (88.6)	0.026
>2	60	32 (53.3)	28 (46.7)		16 (26.7)	44 (73.3)	
Nuclear grade							
1 + 2	101	37 (36.6)	64 (63.4)	0.424	17 (16.8)	84 (83.2)	0.371
3	29	13 (44.8)	16 (55.2)		7 (24.1)	22 (75.9)	
Oestrogen receptor status							
Positive	109	42 (38.5)	67 (61.5)	0.97	18 (16.5)	91 (83.5)	0.192
Negative + unknown	21	8 (38.1)	13 (61.9)		6 (28.6)	15 (71.4)	
HER2 status							
Positive	24	13 (54.2)	11 (45.8)	0.08	8 (33.3)	16 (66.6)	0.038
Negative + unknown	106	37 (34.9)	69 (65.1)		16 (15.1)	90 (84.9)	
Lymphovascular invasion							
Present	89	44 (49.4)	45 (50.6)	<0.001	22 (24.7)	67 (75.3)	0.007
Absent	41	6 (14.6)	35 (85.4)		2 (4.9)	39 (95.1)	
CK19 mRNA in SLN (copies μl^{-1})							
≥2.5 × 10², <5.0 × 10³	57	11 (19.3)	46 (80.7)	<0.001	4 (7.0)	53 (93.0)	0.003
≥5.0 × 10³, <1.0 × 10⁵	39	17 (43.6)	22 (56.4)		8 (20.5)	31 (79.5)	
≥1.0 × 10⁵	34	22 (64.7)	12 (35.3)		12 (35.3)	22 (64.7)	

Abbreviations: CK19 = cytokeratin 19; SLN = sentinel lymph node.

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Table 3 Multivariate analysis of various predictive factors for the presence of non-sentinel node metastases

Factors	Odds ratio (95% CI)	*P*-value
Pathological tumour size		
≤ 2 cm *vs* > 2 cm	2.600 (1.137–5.946)	0.024
Nuclear grade		
1 + 2 *vs* 3	1.051 (0.337–3.279)	0.932
Oestrogen receptor status		
Absent *vs* present	0.996 (0.267–3.716)	0.995
HER2 status		
Negative + unknown *vs* positive	1.855 (0.647–5.321)	0.25
Lymphovascular invasion		
Absent *vs* present	3.472 (1.230–9.799)	0.019
CK19 mRNA in SLN (copies μl^{-1})		
$< 5.0 \times 10^3$ *vs* $\geqslant 5.0 \times 10^3$	3.757 (1.569–8.997)	0.003

Abbreviations: CI = confidence interval; CK19 = cytokeratin 19; SLN = sentinel lymph node.

Table 4 Multivariate analysis of various predictive factors for the presence of four or more node metastases

Factors	Odds ratio (95% CI)	*P*-value
Pathological tumour size		
≤ 2 cm *vs* > 2cm	2.148 (0.766–6.028)	0.146
Nuclear grade		
1 + 2 *vs* 3	0.665 (0.168–2.626)	0.561
Oestrogen receptor status		
Absent *vs* present	0.326 (0.075–1.416)	0.135
HER2 status		
Negative + unknown *vs* positive	2.257 (0.722–7.056)	0.162
Lymphovascular invasion		
Absent *vs* present	4.258 (0.888–20.406)	0.07
CK19 mRNA in SLN (copies μl^{-1})		
$< 1.0 \times 10^5$ *vs* $\geqslant 1.0 \times 10^5$	3.662 (1.301–10.305)	0.014

Abbreviations: CI = confidence interval; CK19 = cytokeratin 19; SLN = sentinel lymph node.

presence of LVI, extracapsular extension, the number of positive SLNs and the size of SLN metastases, have been reported as independent predictors of non-SLN metastases (Chu *et al*, 1999; Degnim *et al*, 2003; Hwang *et al*, 2003; Van Iterson *et al*, 2003; Ozmen *et al*, 2006; van la Parra *et al*, 2011). In this study, we also demonstrated that pathological tumour size, LVI and CK19 mRNA copy number were independent predictors of non-SLN involvement. In particular, the CK19 mRNA copy number had a high odds ratio (3.76). The tumour volume of metastases in the SLN was most frequently identified as a significant predictive factor for non-SLN involvement in many studies. However, these conventional histopathological examinations evaluating the size of metastases are prone to interobserver variability and usually have limited ability for accurately detecting the metastatic volume in LNs, because observations are made on only a portion of the node.

An advantage of the OSNA assay *vs* histological methods is that intraoperative analyses of the whole SLN can be performed in a standardised manner. Several previous studies including ours have reported that the OSNA assay was as accurate as conventional histological examinations for the detection of SLN metastases (Tsujimoto *et al*, 2007; Tamaki *et al*, 2009; Sagara *et al*, 2011; Snook *et al*, 2011). In contrast, there is an inherent difficulty in attempting to validate OSNA assays by comparing them with histopathology of the same SLN because of tissue allocation bias (Snook *et al*, 2011).

Recent studies demonstrated that ALND was not mandatory in the presence of micrometastases (Rayhanabad *et al*, 2010); therefore, the differentiation of micrometastases from macrometastases appears to be important. In the OSNA assay, OSNA + and OSNA + + was considered to be equivalent to micrometastases and macrometastases, respectively, in histology. In this study, the OSNA assay identified micrometastases (OSNA +) in 44.6% (66 of 148) of the SLN-positive patients and 8.9% (66 of 742) of all patients, and it detected micrometastases equivalently to our histological examination results (data not shown). Castellano *et al* (2012) and Cserni (2012) have reported that the rate of micrometastases detected by OSNA was higher than that detected by standard histology. Therefore, the OSNA assay may be at least equivalent or superior to routine histology in the detection of SLN micrometastases. Furthermore, the occurrence of non-SLN metastases in patients with micrometastatic SLNs was 19.3%, which was similar to that obtained in a meta-analysis by Cserni *et al* (2004). As previously reported by Castellano *et al* (2012), our study suggested that the OSNA assay has an almost equivalent reliability compared with gold-standard histological examinations for the prediction of non-SLN metastases.

Recently, the Z0011 trial performed by the American College of Surgeons Oncology Group demonstrated that a subgroup of patients with early-stage breast cancer, with one or two positive SLNs who were treated with breast conserving therapy and adjuvant systemic therapy but did not undergo complete ALND, demonstrated a low locoregional recurrence rate (Giuliano *et al*, 2011). However, the majority of patients in this study had tumours of size T1 and had hormone receptor–positive tumours, which typically have a low risk of reoccurrence. Furthermore, the Z0011 trial did not analyse patients with three or more LN metastases in the SLNs. Our results demonstrated that the frequency of four or more metastases in the LNs was significantly higher in patients with higher CK19 mRNA copy numbers ($\geqslant 1.0 \times 10^5$). Although whether patients with four or more nodes involved could be eligible for the omission of complete ALND may be controversial, higher CK19 mRNA copy number values in SLNs may be an indicator for the selection of treatment, such as radiotherapy, adjuvant chemotherapy and surgical dissection of axillary node. The use of whole SLN analysis by the OSNA assay, when performed in a standardised and objective manner, may be a valuable tool not only for complete ALND decision making but also for further prediction of the axillary node status to assess the risk category of patients who do not undergo complete ALND.

In conclusion, we demonstrated that whole SLN analysis by the OSNA assay is a highly sensitive, specific and reproducible diagnostic technique for predicting additional non-SLN metastases. However, further prospective studies using a larger number of patients are needed to establish a new nomogram, including the results of the OSNA assay.

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