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 enrolled in the study. The association of non-SLN or ≥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 ≥4 LN metastases who were OSNA + and + + was 7.0% and 27.4%, respectively. The cytokeratin 19 (CK19) mRNA copy number (≥5.0 × 10^6; OSNA + +) in the SLN was the most significant predictors of non-SLN metastases (P = 0.003). The CK19 mRNA copy number (≥1.0 × 10^6) in the SLN was the only independent predictor of ≥4 LN metastases (P = 0.014).

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

Keywords: breast cancer; whole sentinel lymph node; one-step nucleic amplification assay; non-sentinel lymph node

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 historical 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 enrolled 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 GFS, 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 (≥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 1 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 χ²-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-SLN 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 $\geq 1.0 \times 10^5$ 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 the frequency of non-SLN metastases in the OSNA + group ($P = 0.024$), LVI ($P = 0.019$) and $\geq 5.0 \times 10^5$ 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 ($\geq 1.0 \times 10^5$) 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

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

| Total number (n = 130) | Non-SLN metastases Present (n = 50) | Absent (n = 80) | P-value | 4 Node metastases 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)        | 16 (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)** |                                      |                |         |                                     |                 |         |
| $\geq 2.5 \times 10^3$, $<5.0 \times 10^4$ | 57                                  | 11 (19.3)      | 46 (80.7) | <0.001                              | 4 (7.0)         | 52 (93.0) | 0.003 |
| $\geq 5.0 \times 10^4$, $<1.0 \times 10^5$ | 39                                  | 17 (43.6)      | 22 (56.4) |                                     | 8 (20.5)        | 31 (79.5)|         |
| $\geq 1.0 \times 10^5$ | 34                                  | 22 (64.7)      | 12 (35.3) |                                     | 12 (35.3)       | 22 (64.7)|         |

Abbreviations: 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 Iersel 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 recurrence. Furthermore, the Z0011 trial did not analyse patients with three or more LNs involved 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 (≥1.0×10³). 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 was 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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