One-step nucleic acid amplification assay for intraoperative prediction of advanced axillary lymph node metastases in breast cancer patients with sentinel lymph node metastasis

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Received July 13, 2015; Accepted October 30, 2015

DOI: 10.3892/mco.2015.694

Abstract. The one-step nucleic acid amplification (OSNA) assay is used to semiquantitatively measure the cytokeratin (CK)19 mRNA copy numbers of each sentinel lymph node (SLN) in breast cancer patients. The aim of the present study was to evaluate whether the diagnosis of ≥4 LN metastases is possible using the OSNA assay intraoperatively. Between May, 2010 and December, 2014, a total of 134 patients who underwent axillary lymph node dissection (ALND) of positive SLNs were analyzed. The total tumor load (TTL) was defined as the total CK19 mRNA copies of all positive SLNs. The correlation between TTL and ≥4 LN metastases was evaluated. Of the 134 patients, 31 (23.1%) had ≥4 LN metastases. TTL ≥$5.4x10^4$ copies/μl evaluated by receiver operator characteristic curve analysis was examined along with other clinicopathological variables. In the multivariate analysis, only TTL ≥$5.4x10^4$ copies/μl was correlated with ≥4 LN metastases (odds ratio = 2.95, 95% confidence interval: 1.17-7.97, P=0.022). Therefore, TTL assessed by the OSNA assay has the potential to be a predictor of ≥4 LN metastases and it may be useful for the selection of patients with positive SLNs in whom ALND may be safely omitted.

Introduction

Axillary surgery in breast cancer has transitioned from level III to level II dissection. Axillary lymph node dissection (ALND) is commonly associated with complications, such as sensory and motor nervous system disorders and edema of the arm. To avoid such complications, sentinel LN biopsy (SLNB) is accepted as a standard technique for clinically node-negative breast cancer patients. SLNB alone (without ALND) is currently the standard course of action for axillary management in patients with negative SLNs (1).

The American College of Surgeons Oncology Group (ACOSOG) Z0011 randomized controlled trial compared overall survival between patients with positive SLNs undergoing SLNB alone and those undergoing SLNB with ALND (2). The trial demonstrated that, for patients with stage T1-2 and ≤2 metastatic SLNs who were treated with breast-conserving surgery followed by whole-breast irradiation and adjuvant systemic therapy, SLNB alone resulted in a similar disease-free and overall survival compared with ALND (2). Furthermore, the International Breast Cancer Study Group (IBCSG) 23-01 trial randomized patients with micrometastases to SLNB alone or ALND groups, and reported a similar disease-free survival for the two patient groups (3). Additionally, the European Organisation for Research and Treatment of Cancer 10981-22023 AMAROS trial compared the axillary recurrence rate between the ALND and axillary irradiation patient groups; both groups consisted of patients with positive SLNs, and the results of the trial demonstrated a similar disease-free and overall survival between the two, similar to the findings of the ACOSOG and IBCSG trials (4). Based on these results, the clinical significance of ALND has been reduced and the selection of patients with positive SLNs in whom ALND may be omitted is attracting increasing attention.

The presence of LN metastases is the most significant prognostic indicator for breast cancer and a major factor in determining adjuvant therapy. Previously, if the presence of LN metastasis was confirmed, postoperative chemotherapy was deemed essential. However, following the St. Gallen Consensus Conference in 2011, the intrinsic subtype of breast cancer has become a more important determinant of adjuvant therapy, rather than the presence of LN metastasis, although the presence of ≥4 LN metastases remains an important indicator, as additional chemotherapy is recommended for such patients, regardless of the cancer subtype (5). Furthermore, when ≥4 LN metastases are identified, irradiation of the supra- and subclavian regions, in addition to the preserved breast, has been reported to improve patient survival as well as local

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Key words: sentinel lymph node, axillary lymph node dissection, one-step nucleic acid amplification, total tumor load, breast cancer

disease control (6,7). Therefore, it is important to determine whether ≥4 LN metastases are present, in order to optimize treatment. Several previous studies have reported a number of methods to predict ≥4 LN metastases (8-11). However, the majority of these relied on factors that were determined post-operatively, and are therefore not widely applied in clinical practice.

The one-step nucleic acid amplification (OSNA) assay is a method for diagnosing LN metastasis through solubilization of LNs and amplification and detection of cytokeratin (CK)19 mRNA. The OSNA assay is able to assess the entire LN, while histopathological examination usually evaluates only the maximum cut surface. The OSNA assay is completed in 30-40 min and is thus suitable for intraoperative diagnosis of SLN metastasis. Several previous studies have reported the usefulness of the OSNA assay for predicting the extent of LN metastasis based on its ability to semi-quantitatively measure CK19 mRNA copy number (12-15,16).

The aim of the present study was to evaluate whether it is possible to intraoperatively diagnose the presence of ≥4 LN metastases in breast cancer patients using the OSNA assay.

Patients and methods

Patients. A total of 621 patients with invasive breast cancer underwent SLNB evaluated by the OSNA assay between May, 2010 and December, 2014 at Kinki University Hospital, Osaka, Japan. Of the 621 patients, 134 who subsequently underwent ALND of the positive SLNs were analyzed. Patients who received neoadjuvant drug therapy, axillary LN sampling alone, and patients with non-invasive cancer, were excluded from this study. Staging was based on the 7th edition of the Union for International Cancer Control TNM classification of malignant tumors (17).

SLN detection. Detection of SLNs was performed using a radioisotope tracer (technetium-99m phytate) and dye (indocyanine green). The day prior to surgery, 85 MBq (0.5 ml) of tracer was injected into the subdermal space in the outer border of the areola. Lymphoscintigraphy was performed 2 h following the injection. During surgery, 5 mg (1 ml) of indocyanine green was injected into the subdermal space in the outer border of the areola and the SLNs were identified using a hand-held gamma probe and dye mapping. SLN metastases were evaluated intraoperatively using the OSNA assay, and ALND was performed when at least one SLN was found to be OSNA-positive (+ or ++, as defined below).

SLN assessment. Whole SLNs were evaluated using the OSNA assay, as previously described (18). SLNs were assessed as OSNA-negative (CK19 mRNA $<2.5\times10^2$ copies/μl), OSNA+ (2.5×10^2 to $<5.0\times10^3$ copies/μl), and OSNA++ ($\geq5.0\times10^3$ copies/μl). When an SLN was assessed as OSNA + inhibition (+I; $\geq2.5\times10^2$ copies/μl in the diluted samples), the patients were excluded from the study, as the reading was not accurate. Total tumor load (TTL) was defined as the total number of CK19 mRNA copies in all positive SLNs. The SLN ratio was defined as the ratio of positive:removed SLNs. Non-SLN tissue sections were evaluated by hematoxylin and eosin staining.

Table I. Patient characteristics (n=134).

Characteristics	No. (%)
Age, years [median (range)]	55.5 (27-81)
Menopausal status	
Premenopausal	51 (38.1)
Postmenopausal	83 (61.9)
Estrogen receptor status	
+	109 (81.3)
-	25 (18.7)
Progesterone receptor status	
+	94 (70.1)
-	40 (29.9)
HER2 status	
+	29 (21.6)
-	105 (78.4)
Breast cancer subtype	
Luminal/HER2-	95 (70.9)
Luminal/HER2+	20 (14.9)
HER2+	9 (6.7)
Triple-negative	10 (7.5)
Type of breast surgery	
Partial mastectomy	70 (52.2)
Total mastectomy	64 (47.8)
Clinical T classification	
cT1	66 (49.3)
cT2	63 (47.0)
cT3	5 (3.7)
Clinical N classification	
cN0	59 (44.0)
cN1 suspected	75 (56.0)
OSNA diagnosis	
+	35 (26.1)
++	99 (73.9)
Histological grade	
1	22 (16.5)
2	44 (32.8)
3	24 (17.9)
Unknown	44 (32.8)
Ki-67	
<20%	46 (34.3)
≥20%	35 (26.1)
Unknown	53 (39.6)
Lymphovascular invasion	
No	53 (39.5)
Yes	79 (59.0)
Unknown	2 (1.5)
Histological type	
Ductal	116 (86.6)
Lobular	11 (8.2)
Other	7 (5.2)

OSNA, one-step nucleic acid amplification. HER2, human epidermal growth factor receptor 2.

Table II. Univariate analysis of variables correlated with ≥4 lymph node metastases.

	LN metastases, no. (%)		
Variables	<4 (n=103)	≥4 (n=31)	P-value
Menopausal status			0.177
Premenopausal	36 (70.6)	15 (29.4)	
Postmenopausal	67 (80.7)	16 (19.3)	
Clinical T classification			0.010
cT1	57 (86.4)	9 (13.6)	
≥cT2	46 (67.6)	22 (32.4)	
Clinical N classification			0.006
cN0	52 (88.1)	7 (11.9)	
cN1 suspected	51 (68.0)	24 (32.0)	
ER status			0.909
+	84 (77.1)	25 (22.9)	
-	19 (76.0)	6 (24.0)	
PR status			0.910
+	72 (76.6)	22 (23.4)	
-	31 (77.5)	9 (22.5)	
HER2 status			0.102
+	19 (65.5)	10 (34.5)	
-	84 (80.0)	21 (20.0)	
TTL (copies/μl)			0.001
$<5.4x10^4$	61 (88.4)	8 (11.6)	
$\geq5.4x10^4$	42 (64.6)	23 (35.4)	
Ki-67			0.144
<20%	39 (84.8)	7 (15.2)	
≥20%	25 (71.4)	10 (28.6)	
Histological grade			0.339
≤2	53 (80.3)	13 (19.7)	
3	17 (70.8)	7 (29.2)	
SLN ratio			0.103
<0.67	34 (91.9)	3 (8.1)	
≥0.67	69 (71.1)	28 (28.9)	
Lymphovascular invasion			0.063
No	45 (84.9)	8 (15.1)	
Yes	56 (70.9)	23 (29.1)	

LN, lymph node; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; SLN, sentinel lymph node; TTL, total tumor load.

Examination of clinical tumor size and axillary nodal status. Clinical tumor size was defined as the largest tumor size measured by magnetic resonance imaging, ultrasonography (US) or computed tomography (CT). Clinical axillary LN metastasis diagnosis was established using contrast-enhanced CT and US. If an LN was diagnosed as metastatic using fine-needle aspiration biopsy, SLNB was not performed.

Statistical analysis. JMP software version 11 (SAS Institute, Cary, NC, USA) was used for statistical analysis. Univariate and multivariate analyses (logistic regression model) were performed to assess the association of the variables with ≥4 LN metastases. A P-value of <0.05 was considered to indicate statistically significant differences. The accuracy of the TTL was estimated by constructing a receiver operator characteristic (ROC) curve and measuring the area under the curve (AUC).

Results

Clinicopathological characteristics. Of the 621 patients who had SLNs evaluated using the OSNA assay, 170 (27.3%) were OSNA-positive. Of these 170 patients, 61 (35.9%) were found to be OSNA+, 102 (60.0%) were OSNA++, and 7 (4.1%) were

Table III. Multivariate analysis of variables correlated with ≥4 lymph node metastases.

Variables	Odds ratio	95% CI	P-value
Clinical T classification cT1 ≥cT2	2.39	0.98-6.15	0.088
Clinical N classification cN0 cN1 suspected	2.31	0.88-6.57	0.055
TTL (copies/μl) <5.4x10^4 ≥5.4x10^4	2.95	1.17-7.97	0.022

CI, confidence interval; TTL, total tumor load.

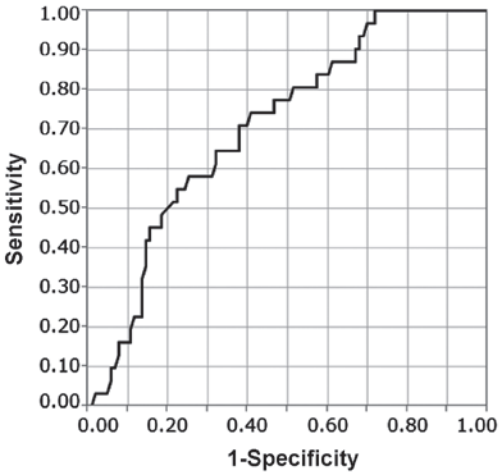

Figure 1. Receiver operator characteristic curve of the total tumor load. The area under the curve was 0.708.

OSNA + I. Of the OSNA-positive patients, 134 (35 OSNA+ and 99 OSNA++) who underwent ALND were eligible for inclusion in our study (Table I). A total of 31 patients had one OSNA+ SLN, 4 patients had two OSNA+ SLNs, 62 patients had one OSNA++ SLN, 14 patients had two OSNA++ SLNs, 3 patients had three OSNA++ SLNs, 17 patients had one OSNA+ and one OSNA++ SLNs, and 3 patients had one OSNA+ and two OSNA++ SLNs. The median number of dissected LNs was 18.3 (range, 5-43). Of the 134 patients, 31 (23.1%) had ≥4 LN metastases.

Association between TTL and ≥4 LN metastases. The association between TTL and ≥4 LN metastases was evaluated using ROC curve analysis (Fig. 1). The median of TTL was 4.5x10^4 copies/μl (2.5x10^2-6.0x10^6). The AUC of the ROC curve was 0.708 and the TTL cut-off was 5.4x10^4 copies/μl. Of the patients with TTL <5.4x10^4 copies/μl, 8 (11.6%) had ≥4 LN metastases and, of the patients with TTL ≥5.4x10^4 copies/μl, 23 (35.4%) had ≥4 LN metastases (Table II). With a TTL cut-off of 5.4x10^4 copies/μl, the sensitivity, specificity, positive and negative predictive values were 74, 59, 35 and 88%, respectively.

Variables correlated with ≥4 LN metastases. The association between clinicopathological variables and ≥4 LN metastases was evaluated. In the univariate analysis, clinical T classification (T1 vs. ≥ T2, P=0.01), clinical N classification (P=0.006), and TTL ≥5.4x10^4 copies/μl (P=0.001) were correlated with ≥4 LN metastases (Table II). In the multivariate analysis, TTL ≥5.4x10^4 copies/μl was the only factor significantly correlated with ≥4 LN metastases (OR=2.95, 95% CI: 1.17-7.97, P=0.022; Table III).

Discussion

A number of previous studies have reported on the prediction of non-SLN metastasis in SLN-positive patients. Osako *et al* demonstrated that SLN copy number, number of macrometastatic SLNs and lymphovascular invasion were significant factors for the identification of non-SLN metastasis (15). According to a meta-analysis of studies investigating non-SLN metastasis prediction, size of SLN metastasis >2 mm, extracapsular extension in SLNs, ≥2 positive SLNs, ≤1 negative SLN, tumor size >2 cm, ratio of positive SLNs >50% and lymphovascular invasion in the primary tumor, have been reported to be strongly correlated with non-SLN metastasis (19).

It was previously reported that ~50% of SLN-positive patients have non-SLN metastases (20,21). However, even in patients with positive SLNs who did not undergo ALND, but instead received appropriate adjuvant therapy, the locoregional and distant recurrence rates were reported to be 1-2 and 5%, respectively (3), which are significantly lower compared with those in patients with non-SLN metastases. Therefore, even in patients with non-SLN metastases, it is not always necessary to perform ALND. Thus, we hypothesized that it may be more important to predict ≥4 LN metastases rather than non-SLN metastases, and conducted this study to evaluate the factors that predict the presence of ≥4 LN metastases.

Several previous studies have focused on factors associated with ≥4 LN metastases. Maretoja *et al* (8) reported an international multicenter predictive tool for the risk of ≥4 LN metastases in patients with SLN macrometastases and demonstrated that the prevalence of ≥4 LN metastases in each center, the number of positive SLNs, the number of negative SLNs, the histological size of the primary tumor and the presence of extracapsular extension of SLN metastases were strongly correlated with the presence of ≥4 LN metastases. Furthermore, Katz *et al* (9) reported that the number of involved SLNs, extranodal extension, lymphovascular invasion, number of uninvolved SLNs, size of largest SLN metastasis, histology (lobular vs. other), and pathological tumor size were significant factors for identifying ≥4 LN metastases.

In the present study, only TTL was significantly correlated with ≥4 LN metastases. Our analysis demonstrated that pathological factors reported in previous studies (tumor size, histological grade, lymphovascular invasion and histology) were not correlated with ≥4 LN metastases. Although extranodal extension of SLNs was shown to be correlated with ≥4 LN metastases in a number of previous studies, SLNs were solubilized for the OSNA assay in the present study and, for this reason, it was not possible to evaluate extranodal extension. The SLN ratio, which has also been identified as an important

factor in previous studies, was not found to be correlated with ≥4 LN metastases in our analysis.

A number of the aforementioned factors are clinically confounding. For example, as the clinical tumor size and size of SLN metastases differed according to the imaging modality used, there is a limit to their accurate evaluation. Moreover, the majority of these factors, such as extranodal SLN extension, lymphovascular invasion and histological grade, are difficult to assess accurately, pre- or intra-operatively. Therefore, these factors cannot be used during surgery to determine the need for ALND.

In the present study, OSNA diagnosis was significantly correlated with ≥4 LN metastases. Additionally, OSNA diagnosis was one of the factors correlated with ≥4 LN metastases in the univariate analysis (data not shown). As the OSNA assay is an objective method that may be rapidly evaluated during surgery, it is useful for intraoperatively determining the necessity of ALND. An OSNA+ result corresponds to micrometastasis on histopathological examination (18) and, as a previous randomized controlled trial demonstrated, ALND is of no clinical significance with respect to disease-free survival and the survival rate of patients with micrometastases (3,22,23). By contrast, for patients with SLN macrometastases, it is more important to identify those in whom ALND may be safely omitted. Therefore, we consider omission of ALND based on OSNA diagnosis alone to be inadequate.

The usefulness of TTL, assessed by the OSNA assay, was investigated as an alternative to OSNA diagnosis. TTL is considered to reflect the tumor burden in LNs more accurately compared with the number of copies of one SLN. Furthermore, TTL has been previously reported to be useful for predicting the extent of LN metastases. Peg *et al* (16) reported that TTL is an independent predictor of non-SLN metastasis and, if patients have TTL ≥$1.5x10^4$ copies/μl, non-SLN metastasis occurs at a higher frequency. Similar studies have reported a correlation between TTL and non-SLN metastasis (13,15). In the present study, we evaluated the association between TTL and ≥4 LN metastases, and found it to be significant when TTL ≥$5.4x10^4$ copies/μl. Ohi *et al* (14) investigated the correlation between ≥4 LN metastases and the maximum copy number of SLNs, and reported that $1.0x10^5$ copies/μl were correlated with ≥4 LN metastases; however, they only evaluated one SLN, namely the one with the maximum number of copies. We consider that the total copy number of all the SLNs is more significantly correlated with the extent of LN metastases compared with the maximum copies of one SLN. To the best of our knowledge, the present study was the first to investigate the correlation between TTL and ≥4 LN metastases. We demonstrated that, of the patients with TTL >5.4×104 copies/μl, 23 (35.4%) had ≥4 LN metastases, which suggests that ALND cannot be omitted in these cases.

There major limitation of our study was the AUC of TTL, which was 0.708, and is of moderate accuracy. The development of novel molecular markers associated with LN metastases may improve the accuracy of the TTL.

In conclusion, TTL ≥$5.4x10^4$ copies/μl significantly correlated with ≥4 LN metastases. Therefore, TTL is likely to become an objective tool for intraoperatively deciding the omission of ALND in SLN-positive breast cancer patients. Further studies are required to improve the accuracy of this assessment.

Acknowledgements

The present study was supported in part by grants-in-aid for scientific research from the Japanese Breast Cancer Society.

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