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ORIGINAL ARTICLE

Nomogram to predict non-sentinel lymph node status using total tumor load determined by one-step nucleic acid amplification: first report from Thailand

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Abstract

Background Axillary staging is a significant prognostic factor often used to determine the treatment course for breast cancer. One-step nucleic acid amplification (OSNA) is now the most accepted method for intra-operative assessment of sentinel lymph nodes (SLN) as it can semi-quantitatively determine the tumor burden in these SLN. Axillary lymph node dissection (ALND) may be omitted in patients with limited disease in the axilla. The objective was to create nomogram for prediction of non-sentinel lymph node (NSLN) status using OSNA to avoid unnecessary ALND.

Patients and methods Patients with invasive breast cancer T1–T3 and clinically negative axillary lymph nodes underwent SLN biopsy assessed by OSNA. The patients with positive SLN underwent ALND. Correlations between total tumor load (TTL), clinicopathological parameters, and NSLN status were analyzed by Chi square statistic and logistic regression. Model discrimination was evaluated using receiver-operating characteristic (ROC) analysis.

Results The total number of patients who underwent SLN biopsies was 278. There were 89 patients with positive SLN. NSLNs were positive in 40 patients. Larger tumor size, presence of lymphovascular invasion (LVI) and higher log TTL were independent factors that predicted positive NSLN. TTL can discriminate NSLN status with area under the ROC curve of 0.789 (95% CI 0.686–0.892). Two nomograms using different parameters obtained pre- and post-operatively can predict NSLN involvement with better area under the ROC curve (0.801, 95% CI 0.702–0.900 and 0.849, 95% CI 0.766–0.932, respectively).

Conclusions Nomograms using results obtained via OSNA can predict NSLN status, as well as aid in deciding to omit the use of ALND.

Keywords Breast cancer · Sentinel lymph node · One-step nucleic acid amplification · Nomogram

Introduction

Axillary staging is one of the more important prognostic factors in breast cancer and it is commonly used to determine the treatment course [1, 2]. Sentinel lymph node biopsy (SLNB) is now accepted as standard procedure for axillary staging in clinical node negative breast cancer without inferior outcomes [3, 4]. Delayed axillary lymph node dissection (ALND) after positive SLNB increases operative time and length of total hospital stay [5]. Conventional intra-operative SLN assessment methods, such as frozen sections and touch imprint cytology, have sensitivities of up to 74%. In the presence of micrometastasis, the sensitivity is compromised and leads to false negative results [6].

In the presence of advanced adjuvant treatment, omission of ALND following positive SLNB has been reported

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to be safe [7, 8]. However, approximately 40% of the patients in these two landmark studies had isolated tumor cells or micrometastasis in SLN. The size of SLN metastasis was reported to be correlated with non-sentinel lymph node (NSLN) status [9]. The chance of leaving gross disease in the axilla is increased when there is extensive SLN metastasis. The number of lymph nodes involved by metastasis does not affect prognosis as long as it is limited to SLN [10]. Thus, the spread of tumor cells into NSLN seems to be a key determinant of prognosis. Several studies have tried to identify clinicopathological parameters that can predict NSLN status to avoid unnecessary ALND [11, 12].

The one-step nucleic acid amplification (OSNA) is a molecular technique for the detection of SLN metastasis by reverse transcription amplification of cytokeratin 19 (CK19), a marker of epithelial cells in the SLN. The CK19 copy number indicates the volume of total tumor load (TTL) in the SLN. This method can evaluate the entire SLN and can be performed intra-operatively with a turn-around time of 30–40 min [13–15]. Several cut-off values for CK19 mRNA were proposed to discriminate NSLN status in patients but the values were widely varied among studies [16–19]. Recently, nomograms for the prediction of NSLN status were reported [20, 21]. This study aims to create nomogram for the prediction of NSLN status in early breast cancer patients with clinically negative axillary node using the OSNA technique to avoid unnecessary ALND.

Patients and methods

Patients

Patients with breast cancer were recruited at the Division of Head Neck and Breast Surgery, Department of Surgery, Siriraj Hospital, Mahidol University, Thailand from November 2015 to July 2018. In total, 278 patients aged over 18 years with invasive breast cancer, clinical stages T1–T3, clinically negative axillary lymph nodes, and who were able to give informed consent underwent SLNB. Intra-operative assessment of SLN status was performed by OSNA. The patients with positive SLN underwent ALND. The patients who received neoadjuvant systemic therapy, underwent ALND with less than ten nodes, or had no invasive carcinoma were excluded. Clinicopathological parameters were recorded. Pre-operative imaging including mammography and ultrasonography of the breast was performed in all patients. The tumor size by imaging was the largest size by either mammography or ultrasonography. This study was approved by Siriraj Institutional Review Board (certificate of approval number Si580/2015).

Intra-operative assessment of SLN

The OSNA assay was processed according to the manufacturer's protocol. Before starting to process the lymph nodes, a standard curve, together with a positive and a negative control, was made using reagents from Sysmex®, Japan. SLN were homogenized with disposable Lynoprep blade sets (Sysmex®) and Polytron® PT1300D (Kinematica AG, Switzerland) in 4 ml of glycine buffer (Lynorhag, Sysmex) at 10,000 rpm for 60 s. One ml of the suspension was transferred to a 1.5 ml Eppendorf tube. The tube was then centrifuged at 10,000*g* for 1 min and 200 µl of supernatant was transferred to a new Eppendorf tube. 20 µl of the supernatant was diluted to 1:100 and 1:1,000 with lysis buffer to achieve the final volume of 200 µl and analyzed with the RD100i system (Sysmex®). For each run, a maximum of four nodes were analyzed together with a positive and a negative control. The amount of CK19 mRNA copies in SLNs were calculated from the previously constructed standard curve and reported as (++), (+i), (+), (−) or (−L) for metastasis. The analyzer was calibrated to identify samples containing ≥ 250 copies/µl of CK19 mRNA as positive for metastatic tumor. A positive result was further classified into 2 categories: + and ++. The + was defined as when the CK19 mRNA was ≥ 250 copies/µl and ≤ 5000 copies/µl. The ++ was defined as when the CK19 mRNA was > 5000 copies/µl.

Pathological examination of NSLN

NSLNs were examined by standard hematoxylin and eosin (H&E) staining. The tissue blocks of NSLN were sectioned for 3-micron thickness with an interval of 200 microns for 4–5 levels depended on the size of lymph node.

Statistical analysis

The sample size was calculated using the formula for estimating an infinite population proportion. The expected proportion was 0.8 with an error of 0.08. This gave an estimated sample size of 97 patients. Clinicopathological parameters of the patients were described as frequency, mean ± SD, median, or range according to the types of variable. Univariate analysis of the correlations between log TTL, clinicopathological parameters, and NSLN status was performed by Chi square statistics, Student *t* tests, or non-parametric tests. Multivariate analysis was performed by logistic regression. A discrimination model was evaluated using receiver-operating characteristic (ROC) analysis. The nomograms for prediction of NSLN status were constructed using the coefficient from binary logistic regression. The statistical analysis was performed using SPSS software (International Business Machines Corp., New York, USA) version 21.

Results

The total number of the patients enrolled was 278. There were 89 patients with positive SLN. Mean age at diagnosis of the patients in this group was 54.7 ± 11.7 years. Five patients had bilateral breast cancer. One patient had bilateral positive SLN. Thus, there were 90 SLNB operations with positive SLN and 90 ALNDs were performed. There were 40 ALNDs with NSLN metastasis while 50 ALNDs had no metastasis in NSLN. One patient had bilateral positive SLN but had unilateral NSLN metastasis. Characteristics of the patients with positive SLN are summarized in Table 1. Pre-operative tumor size by imaging was significantly smaller than pathological tumor size with a mean difference of -4.5 ± 12.7 mm, $p < 0.001$. The differences were emphasized in invasive lobular carcinoma, mucinous carcinoma, and metaplastic carcinoma.

Univariate analysis revealed that larger pre-operative tumor size by imaging, larger pathological tumor size, higher number of positive SLN, presence of lymphovascular invasion (LVI), log TTL, and presence of macrometastasis in SLN were all associated with positive NSLN (p value = 0.046, < 0.001, 0.003, < 0.001, < 0.001, and 0.001, respectively). Multivariate analysis by binary logistic regression showed that pathological tumor size, presence of LVI, and log TTL were significantly associated with NSLN metastasis ($p = 0.024$, 0.049, and 0.005, respectively).

Intra-and post-operative predictive models for the prediction of NSLN status were constructed using different parameters. Due to underestimation of pre-operative tumor size by imaging in the patients with pathological cell type other than invasive ductal carcinoma, nine patients were excluded from the analysis for intra-operative predictive model. Binary logistic regression, backward conditional method was used to construct the model. Figure 1 shows predictive models for NSLN metastasis. The nomogram for prediction of NSLN status using the constructed intra-operative predictive model is shown in Fig. 2a. Post-operative predictive model was also constructed using the parameters that can be obtained post-operatively including pathological tumor size, LVI status and log TTL. The nomogram for prediction of NSLN status using the constructed pre-operative predictive model is shown in Fig. 2b. ROC analysis revealed that both intra- and post-operative predictive models can discriminate NSLN status better than the model using CK19 copy number alone (Fig. 3).

Discussion

The OSNA assay has been well established in the intra-operative evaluation of SLN status. Several studies have reported the accuracy of the assay in detecting small amounts of metastasis [22–24]. The advantages of this assay over conventional intra-operative pathological examination (frozen section or touch imprint) are: evaluating the whole lymph node, avoiding tissue sampling errors; no requirement of an experienced pathologist; and the ability to semi-quantitatively evaluate TTL [25]. In this study, we reported nomograms for intra- and post-operative prediction of NSLN metastasis using different parameters obtained pre- and post-operatively.

The size of SLN metastasis affects NSLN metastasis [9]. Using molecular techniques, the size of metastasis in SLN can be evaluated according to CK19 copy number [13]. Several studies have aimed to identify the cut-off levels of CK19 copy number to predict NSLN metastasis. The cut-off levels reported are all different among studies and depended on selected sensitivity and specificity. To avoid any residual disease in the axilla, a high sensitivity is required. However, a high specificity is also required to avoid unnecessary ALND. Nabias et al. reported the cut-off level at 190,000 copies/μl with 73.3% sensitivity to predict NSLN metastasis. This study included quite small number of patients and only 15 (25.86%) patients had positive NSLN [26]. Espinosa-Bravo et al. used a cut-off of 120,000 copies/μl. At this value, they calculated a low sensitivity to predict NSLN metastasis (47%). They conducted subgroup analysis of hormonal receptor positive patients and demonstrated that a cut-off level of 500,000 copies/μl resulted in a sensitivity of 50% [27]. Recently, Terrenato et al. reported the cut-off level at 2150 copies/μl with a sensitivity of 95%. However, at this low cut-off level, the specificity was only 51% [19]. Determination of NSLN status might be complicated because more number of dissected SLN results in less remaining NSLN and the number of dissected SLN directly affects TTL. This leads to a wide range of TTL assessed by OSNA, especially in the patients with a higher number of dissected SLN and with high copy numbers. Another approach that has been proposed by Kubota et al. is to use TTL to predict ≥ 4 nodes involvement instead of discriminating between metastasis in SLN and NSLN [28].

Although the TTL defined by the CK19 copy number is an independent predictor of NSLN metastasis, conventional clinicopathological parameters were also significant factors that correlated with NSLN status. Inclusion of these parameters increases the discrimination power of the predictive model. Nomograms that incorporate clinicopathological parameters have been reported [20, 21].

Table 1 Characteristics of the patients with positive SLN

	All, $n=90$	NSLN negative, $n=50$	NSLN positive, $n=40$	p value
Age (years), mean ± SD	54.7 ± 11.7	56.2 ± 12.0	52.7 ± 11.2	0.164
Imaging tumor size (mm), mean ± SD	26.1 ± 11.2	24.0 ± 9.7	28.7 ± 12.5	0.046
Pathological tumor size (mm), mean ± SD	30.9 ± 15.0	25.0 ± 9.3	38.1 ± 17.5	< 0.001
Primary tumor				
Total mastectomy	78 (86.7)	41 (82.0)	37 (92.5)	0.145
Wide excision	12 (13.3)	9 (18.0)	2 (7.5)	
Number of dissected SLN, median (min–max)	3 (1–7)	3 (1–7)	3 (1–6)	0.373[a]
Number of positive SLN, median (min–max)	1 (1–6)	1 (1–3)	2 (1–6)	0.003[a]
Histologic type				
Invasive ductal carcinoma	82 (91.1)	46 (92.0)	36 (90.0)	0.245
Invasive lobular carcinoma	6 (6.7)	2 (4.0)	4 (10.0)	
Other histologic type	2 (2.2)	2 (4.0)	0 (0.0)	
Tumor grade				
Grade 1	8 (8.9)	4 (8.0)	4 (10.0)	0.946
Grade 2	48 (53.3)	27 (54.0)	21 (52.5)	
Grade 3	34 (37.8)	19 (38.0)	15 (37.5)	
Multifocality				
Absence	64 (71.1)	33 (66.0)	31 (77.5)	0.232
Presence	26 (28.9)	17 (34.0)	9 (22.5)	
Lymphovascular invasion				
Absence	49 (57.0)	37 (74.0)	12 (30.0)	< 0.001
Presence	41 (43.0)	13 (26.0)	28 (70.0)	
Perineural invasion				
Absence	72 (80.0)	43 (86.0)	29 (72.5)	0.112
Presence	18 (20.0)	7 (14.0)	11 (27.5)	
Estrogen receptor				
Negative	17 (18.9)	11 (22.0)	6 (15.0)	0.399
Positive	73 (81.1)	39 (78.0)	34 (85.0)	
Progesterone receptor				
Negative	28 (31.1)	16 (32.0)	12 (30.0)	0.839
Positive	62 (68.9)	34 (68.0)	28 (70.0)	
HER2				
Negative	62 (68.9)	33 (66.0)	29 (72.5)	0.730
Positive	19 (21.1)	11 (22.0)	8 (20.0)	
Equivocal	9 (10.0)	6 (12.0)	3 (7.5)	
Ki67, mean ± SD	33.7 ± 18.4	34.3 ± 18.1	32.8 ± 19.1	0.741
Log TTL, mean ± SD	4.2 ± 1.1	3.8 ± 1.0	4.8 ± 0.9	< 0.001
SLN status				
Micrometastasis	31 (34.4)	25 (50.0)	6 (15.0)	0.001
Macrometastasis	59 (65.6)	25 (50.0)	34 (85.0)	

[a]Non-parametric test

Rubio et al. developed a nomogram that incorporated several parameters including tumor size, LVI status, HER2 status, number of metastatic SLN, and log TTL with an area under the ROC curve of 0.755 [20]. Shimazu et al., however, argued that nomograms using several pathological parameters are not practical for intra-operative decisions. Hence, they did not incorporate LVI status in their analysis [21]. This is despite LVI being one of the more important parameters associated with lymph node involvement [2, 29]. Therefore, incorporation of LVI status in the predictive model results in a better area under the ROC curve. However, disagreement between the core biopsy and surgical specimens was reported and LVI false-negative rate attained by core biopsies was 55% [30]. In the

$$p = \frac{e^z}{1 + e^z}$$

Pre-operative model $z = -6.010 + (0.047 * size) + (1.063 * logTTL)$

Post-operative model $z = -5.935 + (0.059 * size) + (1.094 * LVI) + (0.802 * logTTL)$

Fig. 1 Predictive models for NSLN metastasis. *p* probability of NSLN involvement. For pre-operative model: *size* pre-operative tumor size by imaging (mm); *log TTL* log CK19 copy number. For post-operative model: *size* pathological tumor size (mm); *LVI* defined as 0 in absence of LVI and 1 in presence of LVI; *log TTL* log CK19 copy number

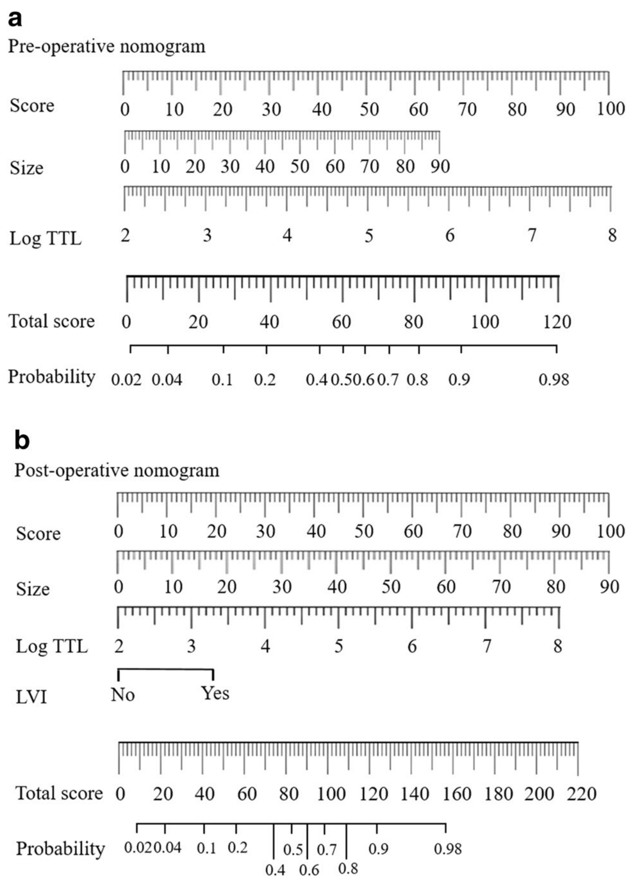

Fig. 2 Nomogram for prediction of NSLN status, **a** pre-operative nomogram and **b** post-operative nomogram

present study, LVI status could not be assessed in most of the core biopsy specimen and cannot be used for intra-operative nomogram.

Rubio et al. used pathological tumor size, while Shimazu et al. used pre-operative clinical tumor size to calculate the probability of NSLN involvement. In our present study, pre-operative tumor size assessed by imaging underestimated pathological tumor size in the patients with the pathological cell type other than invasive ductal carcinoma. The limitation of pre-operative tumor size estimation is that the tumor size is usually underestimated by ultrasonography, especially in invasive lobular carcinoma [31]. Thus, the pre-operative nomogram can be utilized in invasive ductal carcinoma exclusively. The limitation of this study is that this study was conducted in a single institution. The nomogram should be validated before adoption in different institutions.

We proposed the application of a nomogram that combines pre-operative tumor size by imaging and log TTL for intra-operative prediction of NSLN status in invasive ductal carcinoma patients. The another nomogram containing pathological tumor size, LVI status, and log TTL had a better area under the ROC curve and can be applied post-operatively together with the discussion with the patients. ALND might then be omitted in patients with a low probability of NSLN metastasis.

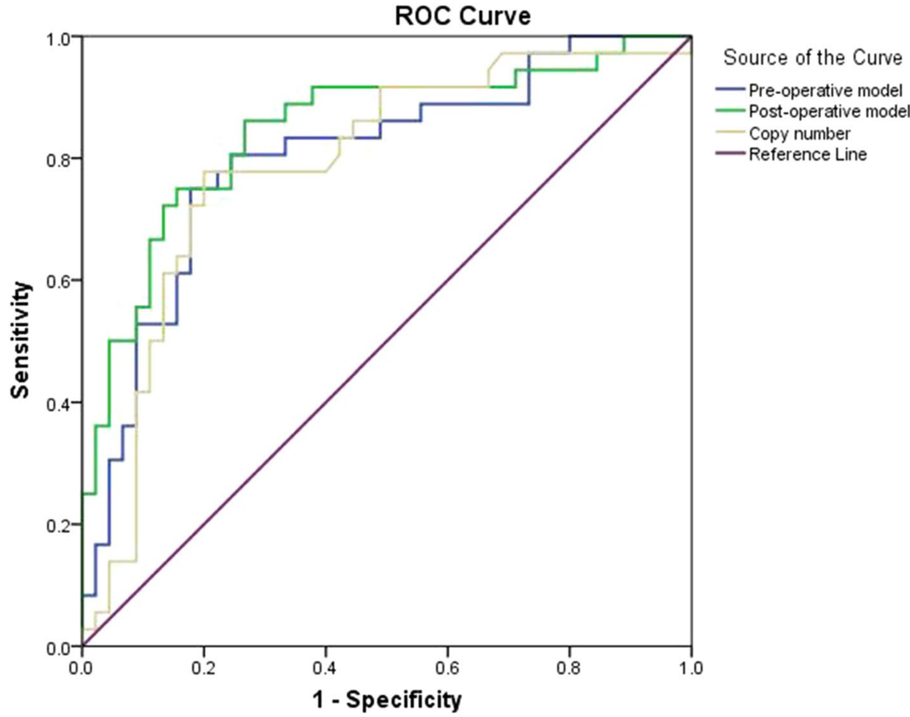

Fig. 3 The pre-operative model for prediction of NSLN metastasis using pre-operative tumor size by imaging and log TTL can discriminate NSLN status with an area under the ROC curve of 0.801, 95% CI 0.702–0.900. The post-operative model using pathological tumor size, LVI status, and log TTL had an area under the ROC curve of 0.849, 95% CI 0.766–0.932. The model using CK19 copy numbers alone discriminates NSLN status with an area under the ROC curve of 0.789, 95% CI 0.686–0.892

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Compliance with ethical standards

Conflict of interest The authors declared no conflict of interest. Sysmex Asia-Pacific Pte Ltd. had no influence in the analysis and interpretation of the results.

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