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A new molecular-based lymph node staging classification determines the prognosis of breast cancer patients

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Background: The one-step nucleic acid amplification (OSNA) assay is a novel molecular method that can detect metastasis in a whole lymph node based on cytokeratin 19 mRNA copy number. This cohort study aimed to establish an OSNA-based nodal staging (pN(mol)) classification for breast cancer.

Methods: The cohort consisted of 1039 breast cancer patients who underwent sentinel node (SN) biopsy using the OSNA assay. Cutoff value of the SN tumour burden stratifying distant disease-free survival (DDFS) was determined, and predictive factors for DDFS and breast cancer-specific survival (BCSS) were investigated. pN(mol) classification of the SN status was defined as: pN0(mol)(sn), SN negative; pN1mi(mol)(sn), SN positive and tumour burden < cutoff-value; and pN1(mol)(sn), tumour burden \geq cutoff-value. Median follow-up time; 68.3 months.

Results: Cutoff value of the SN tumour burden was 2810 copies per μ l. Of the 1039 patients, 798, 95, and 146 had pN0(mol)(sn), pN1mi(mol)(sn), and pN1(mol)(sn) status, respectively. Five-year DDFS and BCSS rates were lower for pN1(mol)(sn) patients than for pN1mi(mol)(sn) patients (87.7% vs 98.8%, $P=0.001$ and 93.1% vs 98.8%, $P=0.044$, respectively). Multivariate analyses revealed the pN(mol) classification was most significant predictor for DDFS and BCSS.

Conclusions: The molecular-based pN classification determines the prognosis of breast cancer patients and could guide therapeutic decision making.

Axillary lymph node status is one of the most powerful prognostic factors in breast cancer (Fisher *et al*, 1983). Accurate and reproducible pathological node staging (pN) classification is an important determinant of the prognosis and therapeutic decision making for breast cancer patients. Sentinel lymph node (SN) biopsy has been the standard axillary staging procedure for clinically node-negative patients since the early 1990s (Lyman *et al*, 2014). To prevent false-negative diagnoses, pathologists began to perform a more detailed evaluation of a fewer amount of lymph nodes, which are most likely to contain metastasis (Giuliano *et al*,

1995). The intensive examination of SNs resulted in an increase in the detection of low-volume metastases (Weaver *et al*, 2009).

The Cancer Staging Manual of the American Joint Committee on Cancer (AJCC), 6th edition, in 2002 (Green *et al*, 2002) classified these low-volume metastases into isolated tumour cells (ITC) and micrometastases. ITC was classified as pN0(i+) with deposits ≤ 0.2 mm, and micrometastasis was classified as pN1mi with deposits > 0.2 mm to ≤ 2 mm. Moreover, in the 7th edition of the AJCC Staging Manual in 2010 (Edge *et al*, 2010), T1 with lymph node spread confined to micrometastasis (pN1mi) was

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downstaged from Stage IIA to Stage IB. However, published studies have reported divergent and conflicting results regarding the prognostic significance of ITC and micrometastasis as defined by the AJCC Staging Manual (Patani & Mokbel, 2011; Salhab *et al*, 2011). These divergent and conflicting results can be attributed to the fact that the AJCC pN classification is based on histopathological findings.

Conventional histopathological examinations are limited in their ability to accurately quantify the total metastatic volume of a lymph node. Even if a node is step-sectioned and histologically evaluated at each cut surface, the information gathered is incomplete, since only a small part of the node is analysed. Furthermore, histopathological examination procedures for SNs are non-standardised, and the inter-observer reproducibility of measuring metastatic tumour volume is low (Cserni *et al*, 2005).

The one-step nucleic acid amplification (OSNA) assay (Sysmex, Kobe, Japan) was developed to overcome the limitations of histopathological examination of lymph nodes. This assay can assess the whole lymph node and yields the quantitative data in the form of the cytokeratin 19 (CK19) mRNA copy number (Tsujimoto *et al*, 2007). Calibration and validation studies (Tamaki *et al*, 2009; Tsujimoto *et al*, 2007) have provided reasonable evidence that the CK19 mRNA copy numbers detected by the OSNA assay are good estimates of macrometastasis, micrometastasis, and negative, as defined by the AJCC Staging Manual (Edge *et al*, 2010). We have shown that the OSNA whole-node assay detects more cases of SN metastases, particularly micrometastasis, than conventional histological examinations (Osako *et al*, 2011b; Osako *et al*, 2012).

Therefore, the OSNA whole-node assay would enable us to more accurately and reproducibly determine the prognosis of breast cancer patients than the current AJCC pN classification based on histopathological examinations. In order to establish a new molecular-based pN (pN(mol)) classification using the OSNA assay, this retrospective cohort study was designed to determine and validate the prognostic cutoff values of the metastatic tumour burden in the SN, as quantified by the CK19 mRNA copy number.

PATIENTS AND METHODS

Patients. The retrospective cohort included patients with clinically and radiologically node-negative invasive breast cancer who underwent SN biopsy and whose whole SNs were examined using the OSNA assay at the Cancer Institute Hospital (Tokyo, Japan) between April 2009 and June 2011. The exclusion criteria were as follows: (1) SN mapping without the use of a radioisotope tracer, (2) bilateral breast cancer, (3) heterochronous ipsilateral breast cancer recurrence, (4) previous excision of a primary tumour, and (5) neoadjuvant drug therapy. The written general consent was obtained from each of the patients, and this study was approved by the Institutional Review Board of the Cancer Institute Hospital.

The pathological tumour staging (pT) classification was classified according to the 7th edition of the AJCC Staging Manual (Edge *et al*, 2010). Hormone receptor status and human epidermal growth factor receptor-2 (HER2) status were defined according to the American Society of Clinical Oncology/College of American Pathologists guidelines (Hammond *et al*, 2010; Wolff *et al*, 2013). The labelling index value for Ki67 was evaluated by estimating the % of positive nuclei within the areas of highest labelling density.

SN biopsy. Lymphoscintigraphy using ^{99m}Tc -phytate was performed one day prior to the surgery, and a vital blue dye, indigo carmine, was injected into the peri-tumoural space or areola at the time of surgery. Before surgery for the primary tumour, the SNs were identified using a hand-held gamma-probe with guidance from staining of the vessels and nodes. Radioactive and/or blue

nodes were considered to be SNs and were excised. When the SN(s) were positive, additional axillary lymph node dissection was performed.

OSNA assay. Each of the whole lymph nodes were homogenised with 4 ml lysis buffer solution (Lynorhag; Sysmex) and centrifuged at 10 000 g at room temperature (Tsujimoto *et al*, 2007). A total of 2 μl supernatant was analysed with an automated molecular detection system, the RD-100i System (Sysmex) and the LymoampBC Kit (Sysmex). The degree of amplification was detected on the basis of a reaction by-product, pyrophosphate. The resultant change in turbidity upon precipitation of magnesium pyrophosphate was then correlated with the CK19 mRNA copy number per μl of the original lysate via a standard curve established beforehand with three calibrators containing different CK19 mRNA copy numbers. Standard positive and negative control samples were used for quality assurance in every assay run. Lymph nodes that exceeded the specified maximum weight of 600 mg were cut into two or more pieces and processed separately.

The number of CK19 mRNA copies per μl in the measurement sample and the 1:10 diluted sample were calculated; the result was determined based on these copy numbers. When the reaction was inhibited in the measurement sample, the copy numbers in the diluted sample were employed for this determination. Lymph nodes with CK19 mRNA <250 copies per μl were considered to be negative, including ITC, and lymph nodes with CK19 mRNA 250–5000 copies per μl or ≥ 5000 copies per μl were considered to be equivalent to AJCC micrometastasis or macrometastasis, respectively (Tsujimoto *et al*, 2007). Tsujimoto *et al*. determined these cutoff values by measuring CK19 mRNA in 2³-mm³-size metastatic tumour tissues and histopathologically positive and negative lymph nodes using the OSNA assay. In their clinical validation study, half of each lymph node was assessed by the OSNA assay and the remaining half was paraffin embedded for three-level histological examination with CK19 immunostaining, and an overall concordance rate between those methods was 98.2% (Tsujimoto *et al*, 2007).

pN classifications of the SN status. Two pN classifications of the SN status were evaluated: that is, the AJCC pN classification and the new pN(mol) classification. For applying the OSNA assay results to the AJCC pN classification (Edge *et al*, 2010), each of the SNs was classified into negative, micrometastasis or macrometastasis using the original cutoff values (<250, 250–5000, and ≥ 5000 copies per μl , respectively).

For defining the new pN(mol) classification, a cutoff value for the tumour burden in the SN stratifying distant disease-free survival (DDFS) was determined. When more than one SN specimen was examined, the total copy number was considered as the tumour burden. The pN(mol) classification of the SN status was defined as follows: pN0(mol)(sn), SN negative; pN1mi(-mol)(sn), SN positive and the total copy number <cutoff-value; and pN1(mol)(sn), SN positive and the total copy number \geq cutoff-value.

Detection of non-SN metastasis. Non-SNs in the axillary dissection materials were examined with routine histology or the OSNA assay according to the study period. Between April 2009 and September 2009, non-SN metastasis was detected with single-section histopathology. Between September 2009 and June 2011, each non-SN was examined with the OSNA whole-node assay for clinical research (Osako *et al*, 2011a; Osako *et al*, 2013).

Adjuvant treatment and follow-up. After the surgery, the patients received a combination of routine adjuvant treatments according to international standards and the national guideline, based on tumour characteristics, including hormone receptor status, HER2 status, lymph node status, and surgical treatment.

Patients were followed-up with a clinical examination, mammography, and ultrasonography.

Statistical analyses. To compare the frequencies of non-SN metastasis, we performed two-sample test for equality of proportions with continuity correction.

DDFS and breast cancer-specific survival (BCSS) were used as prognostic endpoints. DDFS was defined as the period from surgery to distant metastasis of breast cancer, and BCSS was defined as the period from surgery to breast cancer death. The cumulative survival rates were calculated by the Kaplan–Meier method.

To define the new pN(mol) classification, an optimal cutoff value for the tumour burden in the SN was determined according to the maximally selected log-rank statistics analysis. For validating the prognostic impact of the pN(mol) classification on DDFS and BCSS, univariate log-rank tests and multivariate Cox proportional hazards regression models were used. Multivariate analysis was used for the significant factors from the univariate analyses, and the optimal models were selected by Akaike's Information Criterion. *P*-values <0.05 were considered to be significant, and the confidence intervals (CI) were set at the 95% level. All statistical analyses were performed with R statistical software (version 3.3.2, <http://www.r-project.org/>).

RESULTS

Patient characteristics. Between April 2009 and June 2011, 1296 patients with invasive breast cancer underwent SN biopsy using the OSNA whole-node assay, and 1039 of them did not meet the exclusion criteria. The demographic characteristics of the entire cohort are presented in Table 1. Of the 1039 patients, 319 (30.7%) received adjuvant cytotoxic chemotherapy, and 117 (36.7%), 4 (1.3%), 194 (60.8%), and 4 (1.3%) of them received anthracycline-containing regimen alone, taxane-containing regimen alone, both the anthracycline and taxane, and other regimens, respectively. The median follow-up time was 68.3 months (range, 2.0–85.8).

pN(mol) classification of the SN status. The best discriminative cutoff value of the metastatic tumour burden for stratifying DDFS was 2810 copies per μ l (Figure 1). Using this cutoff value, of the 1039 patients, 798 (76.8%), 95 (9.1%), and 146 (14.1%) had pN0(mol)(sn), pN1mi(mol)(sn), and pN1(mol)(sn) status, respectively. The demographic characteristics of each category are presented in Table 1.

Non-SN status of SN-positive patients. Apart from one patient with pN1mi(mol)(sn) disease, all of the SN-positive patients underwent additional axillary dissection. Macrometastases in non-SN were more frequently found in pN1(mol)(sn) patients than in pN1mi(mol)(sn) patients (47 out of 146, 32.2% vs 13 out of 94, 13.8%, *P* = 0.002) (Figure 2). However, there was no difference in the frequency of non-SN metastasis (micro- and macrometastasis) between pN1mi(mol)(sn) patients and pN1(mol)(sn) patients (41 out of 94, 43.6% vs 73 out of 146, 50.0%, *P* = 0.40). Regarding the examination method for non-SN, the OSNA assay detected more cases of non-SN micrometastasis than the single-section histology.

Distant disease-free survival. Five-year DDFS rates were lower for pN1(mol)(sn) patients than for pN0(mol)(sn) patients (87.7% vs 98.0%, hazard ratio (HR) 6.94 (3.50–13.77), *P* < 0.001) and for pN1mi(mol)(sn) patients (87.7% vs 98.8%, HR 12.95 (1.73–95.00), *P* = 0.001) (Figure 3A). There was no significant 5-year DDFS difference between pN1mi(mol)(sn) and pN0(mol)(sn) patients (98.8% vs 98.0%, HR 0.55 (0.07–5.15), *P* = 0.56).

In the univariate analysis, in addition to the pN(mol)(sn) status, DDFS was significantly related to breast surgery procedure, pT classification, grade, hormone-receptor status, Ki67 labelling index,

AJCC pN(sn) classification, positive SN ratio, non-SN status, and adjuvant cytotoxic and endocrine therapies (Table 2). On the other hand, DDFS was not significantly related to age, lymphovascular invasion, HER2 status, and adjuvant anti-HER2 therapy. In the multivariable analysis, pN(mol)(sn) classification, progesterone receptor status, pT classification, and Ki67 labelling index remained significant (Table 3).

Breast cancer-specific survival. Five-year BCSS rates were lower for pN1(mol)(sn) patients than for pN0(mol)(sn) patients (93.1% vs 99.4%, HR 10.06 (3.37–30.02), *P* < 0.001) and for pN1mi(mol)(sn) patients (93.1% vs 98.8%, HR 6.30 (0.80–49.70), *P* = 0.044) (Figure 3B). There was no significant 5-year BCSS difference between pN1mi(mol)(sn) and pN0(mol)(sn) patients (98.8% vs 99.4%, HR 1.70 (0.20–14.54), *P* = 0.63).

In the univariate analysis, in addition to the pN(mol)(sn) status, BCSS was significantly related to pT classification, grade, lymphovascular invasion, hormone-receptor status, Ki67 labelling index, AJCC pN(sn) classification, positive SN ratio, non-SN status, and adjuvant endocrine therapy (Table 2). On the other hand, BCSS was not significantly related to age, breast surgery procedure, HER2 status, and adjuvant cytotoxic and anti-HER2 therapies. In the multivariable analysis, pN(mol)(sn) classification, progesterone receptor status, and pT classification remained significant (Table 3).

DISCUSSION

As far as we know, the present study is the first report to establish a new molecular-based lymph node staging classification for breast cancer without using any histopathological examinations. The new pN(mol) classification is characterised by a total quantification of the metastatic tumour burden in the SN based on the CK19 mRNA copy number using the OSNA whole-node assay, which can more accurately and reproducibly evaluate the metastatic volume than conventional histopathological examinations. Using the pN(mol) classification, pN1(mol)(sn) patients showed a significantly worse prognosis than pN0(mol)(sn) or pN1mi(mol)(sn) patients, and the SN status was the most powerful prognostic factor in early-stage breast cancer.

The prognostic cutoff value was set at 2,810 copies per μ l of CK19 mRNA, which is within the range of the tumour burden equivalent to AJCC micrometastasis (250–5000 copies per μ l) (Tsujimoto *et al*, 2007). Therefore, patients with AJCC micrometastasis can possibly be divided into a good prognosis group and a poor prognosis group according to the metastatic volume. However, conventional histopathological examinations are limited in their ability to accurately and reproducibly evaluate the micrometastasis in a lymph node. This may be attributed to the divergent and conflicting results of the prognostic significance of AJCC micrometastasis in previous studies (Salhab *et al*, 2011). On the other hand, the OSNA assay can accurately and reproducibly evaluate the small metastatic volume, thus the pN(mol) classification could precisely determine patient's prognosis.

A Spanish group has recently proposed the prognostic cutoff value of 25 000 copies per μ l (Peg *et al*, 2017), which is higher than the cutoff value obtained in the present study (2810 copies per μ l). The Spanish group determined the cutoff value by quartering the tumour burdens and testing each of the quartile points for statistical significance. In the present study, however, the optimal cutoff value was more precisely determined by selecting the minimum *P*-value of all possible cutoff points shown in the Figure 1. Applying the Spanish cutoff value to the Figure 1, this cutoff value is statistically significant (*P* = 8.31e⁻⁸), but the cutoff value of 2810 copies per μ l is more significant for stratifying patient survival (*P* = 1.18e⁻¹¹) than the Spanish cutoff value. In

Table 1. Patient characteristics according to the molecular-based pN classification of the sentinel node status

Characteristic	No.	%	pN0(mol)(sn)		pN1mi(mol)(sn)		pN1(mol)(sn)	
			No.	%	No.	%	No.	%
No. of patients	1039	100.0%	798	(76.8%)	95	(9.1%)	146	(14.1%)
Age (years)								
≤ median (25–53)	537	51.7%	416	52.1%	43	45.3%	78	53.4%
> median (54–89)	502	48.3%	382	47.9%	52	54.7%	68	46.6%
Breast surgery								
Partial mastectomy	652	62.8%	525	65.8%	56	58.9%	71	48.6%
Total mastectomy	387	37.2%	273	34.2%	39	41.1%	75	51.4%
pT classification								
pT1a	238	22.9%	218	27.3%	14	14.7%	6	4.1%
pT1b	232	22.3%	194	24.3%	21	22.1%	17	11.6%
pT1c	400	38.5%	288	36.1%	40	42.1%	72	49.3%
pT2	164	15.8%	96	12.0%	20	21.1%	48	32.9%
pT3	5	0.5%	2	0.3%	0	0.0%	3	2.1%
Nuclear grade								
1	447	43.0%	356	44.6%	40	42.1%	51	34.9%
2	387	37.2%	282	35.3%	40	42.1%	65	44.5%
3	205	19.7%	160	20.1%	15	15.8%	30	20.5%
Lymphovascular invasion								
–	754	72.6%	637	79.8%	56	58.9%	61	41.8%
+	285	27.4%	161	20.2%	39	41.1%	85	58.2%
Oestrogen receptor								
+	841	80.9%	633	79.3%	85	89.5%	123	84.2%
–	198	19.1%	165	20.7%	10	10.5%	23	15.8%
Progesterone receptor								
+	702	67.6%	525	65.8%	74	77.9%	103	70.5%
–	337	32.4%	273	34.2%	21	22.1%	43	29.5%
HER2								
–	914	88.0%	706	88.5%	87	91.6%	119	81.5%
+	121	11.6%	88	11.0%	8	8.4%	27	18.5%
Unknown	4	0.4%	4	0.5%	0	0.0%	0	0.0%
Ki67 labelling index (%)								
≤ median (0.1–15.4)	521	50.1%	412	51.6%	48	50.5%	61	41.8%
> median (15.5–93.8)	518	49.9%	386	48.4%	47	49.5%	85	58.2%
AJCC pN(sn) classification								
pN0(sn)	798	76.8%	798	100.0%	–	–	–	–
pN1mi(sn)	109	10.5%	–	–	95	100.0%	14	9.6%
pN1(sn)	126	12.1%	–	–	–	–	126	86.3%
pN2(sn)	6	0.6%	–	–	–	–	6	4.1%
Positive SN ratio								
≤ 0.5	902	86.8%	798	100.0%	57	60.0%	47	32.2%
0.5–1.0	16	1.5%	–	–	1	1.1%	15	10.3%
= 1.0	121	11.6%	–	–	37	38.9%	84	57.5%
AJCC pN classification (SN + non-SN)								
pN0	798	76.8%	798	100.0%	–	–	–	–
pN1mi	94	9.0%	–	–	82	86.3%	12	8.2%
pN1	109	10.5%	–	–	10	10.5%	99	67.8%
pN2	28	2.7%	–	–	3	3.2%	25	17.1%
pN3	10	1.0%	–	–	–	–	10	6.8%
Adjuvant systemic therapy								
None	193	18.6%	183	22.9%	2	2.1%	8	5.5%
Cytotoxic chemotherapy	319	30.7%	131	16.4%	61	64.2%	127	87.0%
Endocrine therapy	743	71.5%	543	68.0%	82	86.3%	118	80.8%
Anti-HER2 therapy	73	7.0%	41	5.1%	7	7.4%	25	17.1%

Abbreviations: AJCC = American Joint Committee on Cancer; HER2 = human epidermal growth factor receptor-2; SN, sentinel lymph node.

addition, another group has recently proposed a similar cutoff value (2150 copies per μ l) as the present study for predicting non-SN metastasis (Terrenato *et al*, 2017). Thus, we believe that our cutoff value can more accurately stratify patient survival than the Spanish cutoff value.

pN1mi(mol)(sn) patients showed similar prognosis to pN0(mol)(sn) patients, even though pN1(mol)(sn) patients showed significantly worse prognosis than pN0(mol)(sn) or pN1mi(mol)(sn) patients. According to the Dutch MIRROR study,

micrometastases in regional lymph nodes are associated with a reduced disease-free survival rate among early-stage breast cancer patients who did not receive adjuvant therapy; however, adjuvant therapy improved survival (de Boer *et al*, 2009). However, because of the present retrospective study design, it is unknown if pN1mi(mol)(sn) patients show worse survival than pN0(mol)(sn) patients without adjuvant chemotherapy, or if pN1mi(mol)(sn) patients intrinsically show similar survival to pN0(mol)(sn) patients despite of adjuvant chemotherapy.

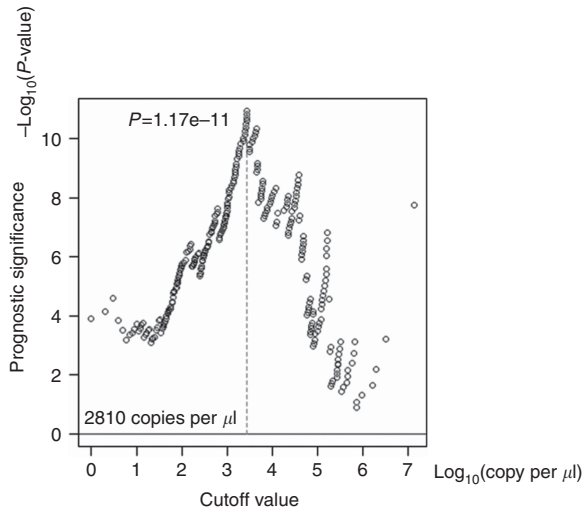


Figure 1. Cutoff value of the metastatic tumour burden in the sentinel node for stratifying distant disease-free survival.

Prospective studies are needed to elucidate the prognostic impact of pN1mi(mol)(sn) status.

Using the pN(mol) classification, the SN status can be the most powerful predictive factor for determining both disease-free and cause-specific survival. After the American College of Surgeons Oncology Group Z-0011 randomised trial (Giuliano *et al*, 2011), additional axillary dissection can be omitted for clinically node-negative patients who have one or two positive SNs and who are receiving adjuvant systemic chemotherapy and breast-conserving surgery with tangential irradiation (NCCN, 2016). Therefore, the pN(mol) classification of the SN status is useful to predict the prognosis of patients who omit additional axillary dissection after positive SN biopsy.

However, the non-SN status in axillary dissection material was not a prognostic factor in the multivariate analysis. This may be because non-SN metastasis, especially macrometastasis, is strongly associated with the SN tumour burden, quantified using the OSNA assay (Osako *et al*, 2013), and the pN(mol) classification of the SN status can be a cofounding factor for the association between non-SN status and prognosis. However, one study has shown that identifying tumour spread to non-SNs beyond SNs appears to be

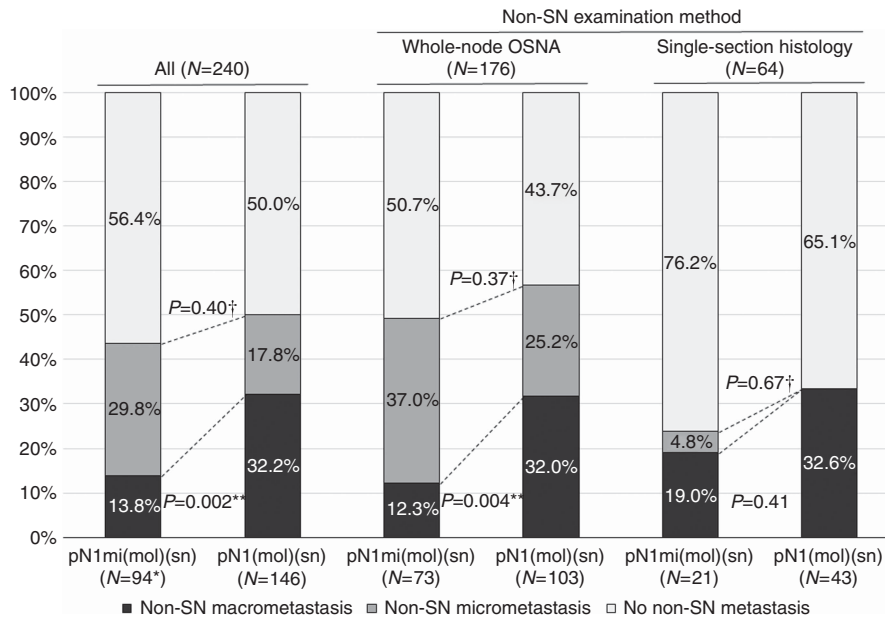


Figure 2. The non-SN status of pN1mi(mol)(sn) patients and pN1(mol)(sn) patients according to the examination method for the non-SNs. Abbreviations: SN = sentinel lymph node; OSNA = one-step nucleic acid amplification.

*One patient did not undergo additional axillary dissection; ** $P < 0.01$; † difference in the frequency of non-SN metastasis (micro- and macrometastasis).

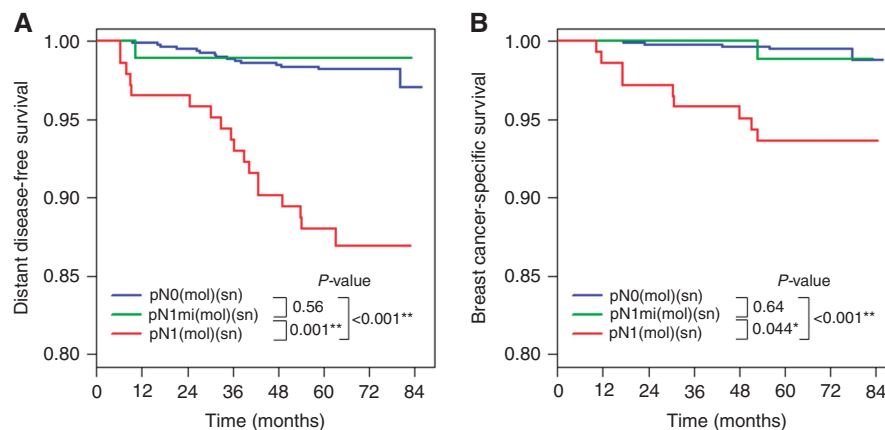


Figure 3. Distant disease-free survival (A) and breast cancer-specific survival (B) according to the pN(mol) classification of the sentinel node status. * $P < 0.05$; ** $P < 0.01$.

Table 2. Univariate analysis of predictive factors for distant disease-free survival and for breast cancer-specific survival

Characteristic	Distant disease-free survival				Breast cancer-specific survival			
	Hazard ratio	95% CI		P	Hazard ratio	95% CI		P
		Lower	Upper			Lower	Upper	
Age								
≤ median	1.00				1.00			
> median	1.79	0.90	3.58	0.094	1.28	0.46	3.52	0.629
Breast surgery								
Partial mastectomy	1.00				1.00			
Total mastectomy	4.17	2.00	8.73	<0.001**	2.58	0.92	7.24	0.063
pT classification								
pT1	1.00				1.00			
pT2 or pT3	5.34	2.73	10.47	<0.001**	5.95	2.16	16.41	<0.001**
Nuclear grade								
1 or 2	1.00				1.00			
3	3.87	1.97	7.58	<0.001**	3.74	1.36	10.33	0.006**
Lymphovascular invasion								
–	1.00				1.00			
+	1.87	0.94	3.70	0.066	3.00	1.09	8.28	0.025*
Oestrogen receptor								
+	1.00				1.00			
–	3.11	1.57	6.16	<0.001**	3.86	1.40	10.65	0.005**
Progesterone receptor								
+	1.00				1.00			
–	3.94	1.95	7.96	<0.001**	4.29	1.47	12.57	0.004**
HER2								
– or unknown	1.00				1.00			
+	1.02	0.36	2.91	0.968	0.54	0.07	4.12	0.545
Ki67 labelling index								
≤ median	1.00				1.00			
> median	4.04	1.76	9.28	<0.001**	6.73	1.52	29.85	0.002**
Molecular-based pN classification								
pN0(mol)(sn) or pN1mi(mol)(sn)	1.00				1.00			
pN1(mol)(sn)	7.30	3.72	14.32	<0.001**	9.42	3.35	26.46	<0.001**
AJCC pN(sn) classification								
pN0(sn) or pN1mi(sn)	1.00				1.00			
pN1(sn) or pN2(sn)	5.67	2.88	11.17	<0.001**	8.01	2.90	22.08	<0.001**
Positive SN ratio								
≤ 0.5	1.00				1.00			
> 0.5	4.77	2.41	9.44	<0.001**	5.79	2.10	15.98	<0.001**
Non-SN metastasis								
–	1.00				1.00			
+	3.57	1.71	7.48	<0.001**	5.72	2.03	16.12	<0.001**
Non-SN macrometastasis								
–	1.00				1.00			
+	3.74	1.55	9.02	0.002 **	6.22	1.98	19.54	<0.001**
Adjuvant systemic therapy								
None	1.00				1.00			
Cytotoxic chemotherapy	2.55	1.30	5.00	0.005 **	2.55	0.92	7.03	0.061
Endocrine therapy	0.48	0.24	0.95	0.028 *	0.33	0.12	0.92	0.025*
Anti-HER2 therapy	0.84	0.20	3.50	0.806	0.00	0.00	0.00	0.284†

Abbreviations: AJCC = American Joint Committee on Cancer; CI = confidence interval; HER2 = human epidermal growth factor receptor-2; SN = sentinel lymph node. *P < 0.05; **P < 0.01; †no event.

an important determinant of patient outcome, and is independent of the number of involved nodes (Jakub *et al*, 2011). We have reported clinical research in which all of the SNs and non-SNs were evaluated by the OSNA whole-node assay without using any histopathological examination (Osako *et al*, 2011a; Osako *et al*, 2013). Follow-up of this cohort may clarify the prognostic impact of the non-SN tumour burden and the total axillary metastatic burden.

There are two potential limitations for the establishment of the pN(mol) classification. First, the present study did not directly compare the prognostic influence of the OSNA-based pN(mol) classification with the current histology-based AJCC pN classification. We found that the AJCC pN classification using the OSNA assay results was less significantly associated with prognosis than the pN(mol) classification. Retrospective or prospective studies are necessary for demonstrating the advantage of the pN(mol)

Table 3. Multivariate analysis of predictive factors for distant disease-free survival and for breast cancer-specific survival

Characteristic	Distant disease-free survival				Breast cancer-specific survival			
	Hazard ratio	95% CI		P	Hazard ratio	95% CI		P
		Lower	Upper			Lower	Upper	
Molecular-based pN classification	7.56	3.41	16.75	<0.001**	7.19	2.46	21.04	<0.001**
Progesterone receptor	3.83	1.88	7.81	<0.001**	3.75	1.27	11.07	0.017*
pT classification	3.44	1.65	7.16	<0.001**	3.12	1.09	8.95	0.034*
Ki67 labelling index	2.49	1.07	5.80	0.034*	4.15	0.92	18.66	0.063
Cytotoxic chemotherapy	0.52	0.24	1.15	0.106	–	–	–	–

Abbreviation: CI = confidence intervals. *P<0.05; **P<0.01.

classification over the histology-based AJCC pN classification. Second, we adapted the total copy number in the SN for the pN(mol) classification because several previous studies have reported that the total copy number in the SN determines non-SN metastasis (Peg *et al*, 2017; Terrenato *et al*, 2017). However, the maximum copy number in the SN (cutoff value of 2500 copies per μ l) had a similar prognostic impact as the total copy number (unpublished data). The maximum copy number can possibly be used for the pN(mol) classification as a surrogate for the total copy number.

In conclusion, a new molecular-based lymph node staging classification for breast cancer has been established using the prognostic cutoff value of the SN tumour burden, quantified using the OSNA assay. The SN status using the pN(mol) classification is the most powerful prognostic factor in early-stage breast cancer. The pN(mol) classification could more accurately and reproducibly determine the prognosis than the current pN classification, and may help to guide more precise therapeutic decision making for breast cancer patients.

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CONFLICT OF INTEREST

FA has received consulting fee from Sysmex Corporation paid to the Cancer Institute. The remaining authors declare no conflict of interest.

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