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Prediction of non-sentinel lymph node metastasis in early breast cancer by assessing total tumoral load in the sentinel lymph node by molecular assay

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Abstract

Introduction: The one-step nucleic acid amplification (OSNA) is a molecular procedure that yields a semiquantitative result for detection of nodal metastasis. Size of metastasis in the sentinel lymph node (SLN) by conventional histology has been described as a predictive factor for additional axillary metastasis. The objective of this study is to quantify intraoperatively the total tumoral load (TTL) in the positive SLNs assessed by OSNA and to determine whether this TTL predicts non-SLN metastasis in patients with clinically node negative early stage breast cancer.

Methods: 306 patients with cT1-3N0 invasive breast cancer who had undergone intraoperative SLN evaluation by OSNA were included. TTL was defined as the addition of CK19 mRNA copies of each positive SLN (copies/μL).

Results: TTL was a predictive factor of additional non-SLN metastasis in the complete axillary lymph node dissection (cALND) (OR, 1.67; 95% CI, 1.18–2.35). In the multivariate analysis, the TTL was a predictor of non-SLN metastasis in HR positive patients (OR, 1.69; 95% CI, 1.19–2.41). In our cohort of patients, with a TTL $\leq 1.2 \times 10^5$ copies/μL, there was a specificity of 85.3% and negative predictive value (NPV) of 80%. If we consider only the HR positive patients, with a TTL $\leq 5 \times 10^5$ copies/μL there was a specificity of 86.7% and NPV of 83.7%.

Conclusions: TTL assessed by OSNA assay predicts for additional non-SLN metastasis and this intraoperative tool can help guiding decisions on performing a cALND in breast cancer patients.

Keywords: Breast cancer; Whole sentinel lymph node; One-step nucleic amplification assay; Non-sentinel lymph node

Introduction

Sentinel lymph node (SLN) biopsy is a standard procedure for patients with clinically node-negative early-stage breast cancer.[1] Axillary lymph node status remains the most powerful prognostic factor in breast cancer patients and nodal tumor load is a continuous variable of prognosis.[2,3] Patients with SLN micrometastasis have an incidence of non-SLN metastasis that varies from 5% to 25% and this incidence increases when the SLN contains a macrometastasis up to 40%–60%.[4,5] Recently, it has been reported that the number of metastatic axillary lymph nodes does not affect outcome as long as all disease is confined to the SLN.[6] Therefore, multiple studies have tried to identify variables that are predictive of non-SLN metastasis in an attempt to select those patients who can be spared a complete axillary lymph node dissection (cALND). These studies have shown that different pathologic characteristics of the primary tumor and the SLN metastasis are associated with an increased likelihood of additional positive non-SLN.[4,7–9] In

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all this studies the characterization of the SLNs has been based on the maximum size of the metastasis, classifying foci of disease as isolated tumor cells, micrometastasis and macrometastasis.[10]

However, the methods of intraoperative examination of the SLNs are not standardized, are subject to interobserver variability and are limited in its ability to detect metastasis because of the partial evaluation of the nodes.[11] Recently, the One-Step Nucleic Acid Amplification (OSNA) assay (Sysmex Corporation, Kobe, Japan) has been reported as a molecular detection procedure that analyses lymph node metastasis by detection and amplification of cytokeratin 19 (CK19) mRNA.[12,13] It assesses the whole lymph node and yields a semi-quantitative result for detection of nodal metastasis, differentiating micrometastasis from macrometastasis. Several studies have shown that OSNA could accurately detect SLN metastasis in breast cancer at a rate comparable to that of conventional pathological examination.[13–17]

The aim of the present study was to quantify intraoperatively the total tumoral load (TTL) in positive SLNs assessed by OSNA and to determine whether this TTL predicts non-SLN metastasis in patients with clinically node negative early stage breast cancer.

Materials and methods

In this multicentric prospective cohort study, 306 consecutive patients with clinically and ultrasonographically node-negative cT1-3 invasive breast cancer who had undergone intraoperative SLN evaluation by OSNA between February 2010 and August 2011 were included in the study. Exclusion criteria were patients with ipsilateral breast cancer recurrence, neoadjuvant treatment and negative CK19 at the core biopsy in the primary tumor. Data was recorded from two different institutions (Breast Cancer Center at the Hospital Universitario Vall d'Hebron, Barcelona and the Breast Unit at the Hospital Universitario, Salamanca, Spain).

Surgery

The day before surgery, 1.5 mCi/mL of 99mTc was injected subareolar in the breast and SLN was performed as previously described.[18] SLNs were sent fresh to Pathology. Level I and II ALND was performed if the SLNs were positive for micro or macrometastasis intraoperatively by OSNA.

Intraoperative OSNA evaluation

In the Pathology department, the extranodal fat tissue of the SLN was removed. After that, the entire lymph node was homogenized with 4 mL of a lysis buffer solution and centrifuged at 10,000× *g* at room temperature. A 2 μL sample of the supernatant was analyzed with the RD-100i system, an automated gene amplification detection system using a reverse transcription loop-mediated isothermal amplification method with the LynoampBC (Sysmex, Kobe, Japan). The evaluation of the whole lymph nodes can be completed within 30–40 min.[14]

The OSNA technique was performed as described by Tsujimoto et al.[12] Based of the number calculated of CK19 mRNA copies per μL the result was assessed in accordance with the cut off level: macrometastasis (OSNA++) was defined as $>5 \times 10^3$ copies/μL of CK19 mRNA, micrometastasis (OSNA+) as 2.5×10^2 to 5×10^3 copies/μL, and non-metastasis (OSNA−) as $<2.5 \times 10^2$ copies/μL. TTL was defined as the addition of CK19 mRNA copies of each positive SLN (copies/μL).

Permanent histology for non-SLN examination

All non-SLNs were sliced in half along the long axis after formalin fixation. One of the cut surfaces was examined with haematoxylin and eosin (H&E) staining. No immunohistochemical (IHC) staining was used. Axillary lymph nodes were staged according to the American Joint Committee on Cancer.[10]

Pathology of primary tumor

Estrogen receptors (ER), progesterone receptors (PR) and Her2 overexpression were defined according to the ASCO/CAP guidelines recommendations.[19] Proliferation index Ki67 was considered high when Ki67 positive tumor nucleus was greater than 15%.[20]

Statistical analyses

Data collected from each patient included age, tumor size, tumor grade, ER and PR grouped into hormonal receptor (HR), HER2 overexpression, Ki67 status, lymphovascular invasion (LVI), total number of SLN and non-SLN, number of positive and negative SLN and non-SLN, type of SLN metastasis by OSNA and the TTL of CK19 mRNA in copies/μL.

Frequencies were used for qualitative variables. Medians and ranges showed continuous variables. Chi-squared and Mann–Whitney *U* test were used to compare positive and negative SLN.

Stepwise logistic regression was used for risk factors to determine whether there was a difference between the group of non-SLN positive and non-SLN negative patients after a positive SLN. A postfit ROC analyses was carried out to identify the best cut-off of TTL. In all analysis, a *p*-value of <0.05 was taken to indicate statistical significance. The statistical analysis was carried out in SPSS for Windows (SPSS v17.0, Chicago, USA) and STATA 11.2 (StataCorp, College Station, TX, USA).

Results

Patient's characteristics

A total of 616 SLNs from 306 patients were examined. Median age was 62 years (range 28–90). Median clinical tumor size was 16 mm (range 1–80). One hundred and eight patients (35%) had positive SLNs in the intraoperative assessment by OSNA. Patients and disease characteristics divided by negative and positive SLNs are reported in Table 1.

Lymph nodes status

A mean of 1.9 SLNs were removed from each patient in the group with a negative SLN and 2.2 SLNs in the group with positive SLNs ($p = 0.04$). Of 108 patients with positive SLNs, 105 (97%) had a cALND; in two patients with micrometastasis and in one patient with a macrometastasis the cALND was not performed. In seventy-five (71%) patients the SLNs were the only positive nodes. Thirty patients had additional metastasis in the non-SLNs, and in 50% of them there were only one additional metastatic lymph node in the ALND. Median lymph nodes removed in the ALND were 14 (range 6–33). Characteristics of patients with cALND are described in Table 2.

In the group of patients with a positive SLN as the only focus of disease, 37 patients (49%) had a micrometastasis (OSNA+) in the SLN and 38 patients (51%) had a macrometastasis (OSNA++) in the SLN. Of patients with only SLN metastasis, 61 (81%) had one positive SLN and 14 (19%) had two positive SLNs. On the other hand, patients with other non-SLN metastasis in the cALND, 17 (57%) had one positive SLN and 9 (30%) had two positive SLNs. Only 4 patients (13%) of the series had 3 or more positives SLN in the intraoperative evaluation and all of them had another non-SLN metastasis in the cALND.

Table 1
Patient and tumors characteristics by sentinel node (SLN) status by OSNA.

Characteristic	SLN		*p*
	Negative ($n = 198$)	Positive ($n = 108$)	
Age, yr			
Median (range)	63 (32–90)	61 (28–89)	0.76*
<50 yr (%)	53 (27)	37 (34)	0.16[a]
≥50 yr (%)	145 (73)	71 (68)	
Clinical tumor size, mm			
Median (range)	15 (1; 50)	18 (3; 80)	0.00*
Pathological T stage, no. (%)			
pT1mi	1 (1)	0	0.00[a]
pT1a	12 (6)	1 (1)	
pT1b	47 (24)	10 (9)	
pT1c	94 (47)	56 (52)	
pT2	44 (22)	39 (36)	
pT3	0	2 (2)	
Histologic type, no. (%)			
Invasive ductal	168 (85)	90 (84)	0.69[a]
Invasive lobular	20 (10)	10 (9)	
Other	10 (5)	8 (7)	
Histological grade, no. (%)			
I	47 (24)	28 (27)	0.86[a]
II	90 (47)	48 (46)	
III	56 (29)	28 (27)	
Estrogen receptor, no. (%)			
Positive	173 (87)	100 (93)	0.29[a]
Negative	25 (13)	8 (7)	
Progesterone receptor, no. (%)			
Positive	145 (73)	94 (87)	0.03[a]
Negative	53 (27)	14 (13)	
Hormonal receptor status, no. (%)			
Positive	173 (87)	102 (94)	0.050[a]
Negative	25 (13)	6 (6)	
HER2, no. (%)			
Negative	178 (90)	101 (94)	0.28[a]
Positive	20 (10)	7 (6)	
Ki67, no. (%)			
<15%	131 (67)	73 (68)	0.84[a]
≥15%	66 (33)	35 (32)	
Lymphovascular invasion, no. (%)			
No	173 (87)	86 (80)	0.10[a]
Yes	25 (13)	21 (19)	
Missing/Unknown	0	1 (1)	

*p value by Mann–Whitney U test.
[a]p value by chi-squared test.

Univariate and multivariate analysis of non-SLN metastasis

We calculated intraoperatively the TTL number of copies/μL of CK19 mRNA assessed by OSNA in the SLN. The area under the receiver operating characteristics (ROC) curve of TTL was 0.714 (95% IC, 0.59–0.87) (Fig. 1). In the univariate analysis including patient age, tumor size, histological tumor type, tumor grade, HR, HER2, Ki67, LVI status, type of SLN metastasis and number of positive SLNs, TTL was a predictive factor of non-additional axillary metastasis, and this was influenced by the HR status with statistically significant differences (OR, 1.67; 95% CI, 1.18–2.35), $p = 0.003$. In the multivariate analysis only the TTL was a predictor of non-additional axillary metastasis in HR positive patients (OR, 1.69; 95% CI, 1.19–2.41), $p = 0.003$ (Table 3). In our cohort of patients, with the TTL equal or less than 1.2×10^5 copies/μL, there was a specificity of 85% with a negative predictive value of 80% and sensitivity of 47% with a positive predictive value of 56%. If we consider the HR positive patients only, the TTL equal or less than 5×10^5 copies/μL had a specificity of 87% with a negative predictive value of 84%, and a sensitivity of 50% with a positive predictive value of 56%.

Macrometastasis in the SLN had a statistically significant association with additional non-SLN metastasis in the univariate analysis (OR, 2.68; 95% CI, 1.06–6.77; $p = 0.03$) and in the multivariate analysis (OR, 1.93; 95% CI, 1.08–3.43; $p = 0.025$). In the multivariate analysis, the micrometastasis showed no differences statistically

Table 2
Characteristics of patients with a cALND after positive SLN.

Characteristic	Non-SLNs in ALND		p
	Negative ($n = 75$)	Positive ($n = 30$)	
Age, yr			
Median (range)	57 (28–87)	67 (36–84)	
<50 yr (%)	30 (40)	7 (23)	0.10[a]
≥50 yr (%)	45 (60)	23 (77)	
Clinical tumor size, mm.			
Median (range)	17 (3; 42)	20 (9; 80)	0.05*
Pathological T stage, no. (%)			
pT1mi	0	0	0.12[a]
pT1a	1 (1)	0	
pT1b	9 (12)	1 (3)	
pT1c	39 (52)	15 (50)	
pT2	26 (35)	12 (40)	
pT3	0	2 (7)	
Histologic type, no. (%)			
Invasive ductal	62 (83)	26 (87)	0.68[a]
Invasive lobular	7 (9)	3 (10)	
Other	6 (8)	1 (3)	
Histological grade, no. (%)			
I	21 (29)	5 (18)	0.23[a]
II	35 (48)	12 (43)	
III	17 (23)	11 (39)	
Hormonal receptor status, no. (%)			
Positive	74 (99)	25 (83)	0.002[a]
Negative	1 (1)	5 (17)	
HER2, no. (%)			
Negative	71 (95)	27 (90)	0.38[a]
Positive	4 (5)	3 (10)	
Ki67, no. (%)			
≥ 15%	21 (28)	14 (47)	0.06[a]
< 15%	54 (72)	16 (53)	
Lymphovascular invasion, no. (%)			
No	59 (79)	24 (80)	0.81[a]
Yes	15 (20)	6 (20)	
Missing/Unknown	1 (1)	0	
Type of SLN metastasis			
Micrometastasis	37 (49)	8 (26)	0.03[a]
Macrometastasis	38 (51)	22 (73)	
Positive SLN			
1	61 (81)	17 (57)	0.001[a]
2	14 (19)	9 (30)	
≥3	0	4 (13)	
SLN total tumoral load (copies/μL)			
Median (range)	5.2×10^3 (2.8×10^2; 2.5×10^7)	5.6×10^4 (2.6×10^2; 1.2×10^7)	0.006*

*p value by Mann–Whitney U test.
[a]p value by chi-squared test.

significant (OR, 0.63; 95% CI, 0.08–4.52; $p = 0.65$) for additional non-SLN metastasis. The tumor size had a statistically significant association with additional non-SLN metastasis in the univariate analysis (OR, 1.05; 95% CI, 1.00–1.09; $p = 0.03$), but there were no differences in the multivariate analysis (OR, 1.54; 95% CI, 0.63–3.78; $p = 0.34$). The number of positive SLN showed no differences statistically significant (OR, 2.31; 95% CI, 0.85–6.24; $p = 0.09$) for non-additional axillary metastasis in the univariate analysis (Table 3).

Discussion

The one-step nucleic acid amplification assay

The OSNA assay has been reported as a standardized intraoperative technique that studies the whole SLN, thereby avoiding sampling errors. Validation studies published to date are consistent with a reliable quantitative test that allows final decisions related to axillary surgery in patients.[21] OSNA for breast cancer is a simple, reproductible and highly accurate tool for intraoperative assessment of SLN status.[22,23] In fact, some institutions have introduced OSNA in their daily routine practice outside of clinical trials.[13,22,24,25] The sensitivity of the technique is comparable to conventional serial section and it has been shown to be significantly more sensitive than touch smear cytology.[22,25] Results of comparative OSNA tests in different validation series with conventional histology show a sensitivity of 92% and a specificity of 97%. The false-negative rate of the OSNA assay compared with intensive histology is within the generally tolerated 10% limit (8.3%).[21] The semi-quantitative results enabling the differentiation of macrometastasis and micrometastasis render the assay suitable for intraoperative evaluation of SLN.[12,13,16,17]

The total tumoral load implications

The cut-off value of CK19 mRNA copies numbers established by the OSNA assay differentiates between micro- and macrometastasis as proven by Tsujimoto[12] in the technique development clinical study. This cut-off only provides information on the amount of tumoral load of the positive SLN related to the conventional pathologic size of the SLN metastasis. The advantage of OSNA technique over conventional pathology is that allows the semiquantitative evaluation of the total tumoral load in the SLNs when the whole nodes are examined. This is different from the cut-off that we choose for the best predictor of non-SLN metastasis in the axilla. Our study has shown that the

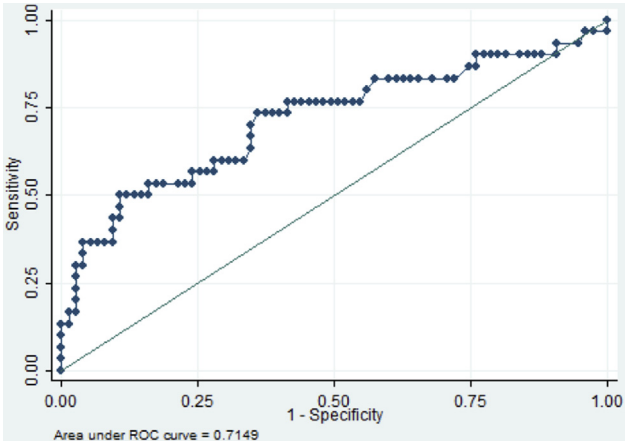

Figure 1. Area under the ROC curve of total tumoral load.

Table 3
Univariable and multivariable associations of prognostic factors with positive non-SLNs.

	Odds ratio (95%CI) Univariable	*p* Univariable	Odds ratio (95%CI) Multivariable	*p* (all variables) Multivariable
Age				
<50	1	0.11		
≥50	2.19 (0.84; 5.74)			
Clinical tumor size	1.05 (1.00; 1.09)	0.03	1.54 (0.63; 3.78)	0.34
Histologic type				
Invasive ductal	1	0.70		
Invasive lobular	1.02 (0.25; 4.26)			
Other	0.40 (0.05; 3.47)			
Histological tumor grade				
I	1	0.24		
II	1.44 (0.44; 4.66)			
III	2.72 (0.79; 9.35)			
Hormonal receptor status				
Negative	1	0.01	1	0.01
Positive	0.06 (0.01; 0.61)		0.06 (0.01; 0.60)	
HER2 status				
Negative	1	0.39		
Positive	1.97 (0.41; 9.40)			
Ki67 status				
<15%	1	0.07		
≥15%	2.25 (0.94; 5.41)			
Lymphovascular invasion				
No	1	0.97		
Yes	0.98 (0.34; 2.84)			
Type of SLN metastasis				
Micrometastasis	1	0.03	0.63 (0.08; 4.52)	0.65
Macrometastasis	2.68 (1.06; 6.77)		1.93 (1.08; 3.43)	0.025
No. Positive SLNs				
1	1	0.09		
2	2.31 (0.85; 6.24)			
SLN TTL[a]	1.67 (1.18; 2.35)	0.003		
SLN TTL[a] of HR positive			1.69 (1.19; 2.41)	0.003

[a] SLN TTL is the total tumoral load of positive sentinel lymph's nodes.

TTL is a predictive tool for additional non-SLN metastasis and based in these results we established the cut-off of the TTL to predict additional non-SLN metastasis that may have clinical utility.

In our cohort of patients, when the TTL was $\leq 1.2 \times 10^5$ copies/μL with a specificity of 85% and a negative predictive value of 80%, 81 (77%) patients will not have additional positive nodes in the ALND. Therefore, only 21% of patients will have additional nodal metastasis in the ALND. And when the TTL was $>1.2 \times 10^5$ copies/μL, 13 (54%) patients will have additional lymph nodes metastasis in the ALND. We tried to identify other prognostic tumor factors that could impact on these results and we found that in HR positive patients, if the cut off of the TTL was $\leq 5 \times 10^5$ copies/μL, with a specificity of 87% and a negative predictive value of 84%, 81 (82%) patients will not have additional positive nodes in the ALND. On the other hand, when the TTL was $>5 \times 10^5$ copies/μL, 10 (56%) patients will have additional lymph nodes metastasis on the ALND. These results suggest that in HR positive patients with greater TTL, the chances of having additional positives nodes in the axilla are not negligible.

Other studies have also reported that the whole sentinel lymph node analysis by a molecular assay predicts axillary node status in breast cancer patients.[26] Ohi et al.[27] have shown that a higher copy number of CK19 mRNA, 1.0×10^5 copies/μL or greater, is significantly associated with four or more axillary LN metastases, although they do not report whether this cut-off of the copy number of CK19 mRNA would be more predictive than the number of positive SLN. In our cohort of patients, the number of positives SLNs (1 or 2) shows no statistical differences in the univariate analysis suggesting that the TTL is more predictive than the number of positives SLN for non-SLN metastasis.

Current perspective on ALND in early breast cancer

Great debate has been generated to identify which patients can avoid an ALND when the SLNs are positive. Van la Parra et al.[8] performed a meta-analysis of predictive factors for non-SLN metastasis in breast cancer patients with a positive SLN. Eight predictive factors were defined: method of detection (H&E), SLN metastasis >2 mm in

size, extracapsular extension in the SLN, >1 positive SLN, 1 negative SLN, ratio of positive SLN >50%, tumor size >2 cm, and LVI in the primary tumor. Also, different nomograms are available to help with the decision of performing a complete ALND and it can be used as a guide for treatment.[7,9] The MD Anderson group has validated a predictive nomogram that incorporates the size of the SLN metastasis.[28] As all the prediction models have reported, one of the most important factors in predicting the axillary metastasis in patients with a positive SLN is the size of the SLN metastasis by conventional histology.[29] The problem is that many of these decisions need to be taken after surgery and when all the pathology of the tumor and SLN is finalized. With the introduction of OSNA, figuring the TTL intraoperatively in the positive SLNs, a model to estimate additional non-SLN metastasis can be generated as a novel predictive tool for patients where the indications of a cALND are still under debate.

Omission of ALND in positive SLN patients

Data from the American College of Surgeons Oncology Group (ACOSOG) Z0011 trial suggest that ALND may no longer be justified for women who have cT1-T2 breast cancer and H&E detected metastasis in the SLN and who are treated with breast-conserving surgery, whole-breast irradiation, and adjuvant systemic therapy.[30] Findings from the Z0011 trial are important and it has been a practice changing since the results were reported. One debate concerning the Z0011 trial is the applicability of the results to subsets of patients who were underrepresented or undefined in the trial. All the issues related to the trial have been extensively discussed in many reports but even groups participating in the trial have reported that some patients who qualified for omission of ALND by strict Z0011 eligibility criteria still undergo ALND.[31] Patients who meet Z0011 eligibility criteria but may not completely meet the characteristics of those who enrolled on the trial or groups that were underrepresented in the trial such as young patients, patients with lobular histology, hormone receptor negative tumors, or HER2-positive tumors are a group of patients where ALND is still performed after a positive SLN in many institutions. Nomograms are used to help in the decision to complete the ALND in these patients.[31] With the intraoperative predictive value of the total tumoral load we have another data to assess the likelihood of non-SLN positivity and in some cases, it can help to guide the surgical decision. Although intraoperative sentinel node biopsy have been reduced since the results from the Z011, in fact it has been reported to have decreased from 69% in the pre-Z0011 era to 29%, surgeons still are more likely to perform intraoperative SLN assessment for patients with younger age, higher clinical T stage, ductal histology and high-grade tumors.[31] If we consider the intraoperative assessment of the SLN, the use of molecular assays provides intraoperative predictive information by itself. One of the differences among conventional intraoperative assessment of the SLN and the OSNA method is that the OSNA method is definitive so no postoperative histologic assessment is done. We consider that the decision of performing a cALND in some group of patients should be based on as much information as we can provide when there are still issues to debate, realizing that many patients with early breast cancer are not benefiting anymore from a cALND. That is why we consider that intraoperative assessment of total tumoral load is still useful as it provides with additional predictive information to be considered in some groups of patients, and if, in theses cases, the results are intraoperatively recorded, the decision can be made at that time.

The TTL as a predictive tool

The discrimination power of the TTL assessing by OSNA was quantified with the ROC curve. This discrimination, as measured by the area under the curve (AUC), provides a measure of whether the relative ranking of individual predictions is in the correct order. The overall predictive accuracy of the TTL, as measured by the AUC was 0.71 (95% CI, 0.59−0.83). The AUC in our study considering only the TTL compares favorably with AUC from other nomograms developed to predict non-SLN metastasis in patients with a positive SLN.[9,28]

When assessing the TTL in our cohort of patients, only 21% of patients would have additional non-SLN metastasis if a cALND would have not been performed. Whether metastasis in the ALND are the same assessed by OSNA or conventional histology, Castellano et al. showed that the rate of metastasis in the ALND after a positive SLN was not significantly different when comparing patients in the OSNA group and in the standard histology group.[24] It has also been reported that the rate of micrometastases detected by OSNA was higher than that detected by standard histology,[21,24] the occurrence of non-SLN metastases in patients with micrometastatic SLNs in our series was 17.7%, which was similar to that obtained in a meta-analysis by Cserni et al.[32]

Our study has the limitations of a retrospective study. Other limitation is the clinical implications of the TTL in the way we surgically manage patients with positive SLNs. Taking into account that there are a limited number of positives SLNs to determine the need of an ALND, further studies in our group are on the way to assess if the TTL is a predictor of non-SLN metastasis better than the number of affected SLNs. A nomogram that includes the TTL for predicting non-SLN axillary metastasis is also being explored.

In the last years, gene expression analysis that characterized the variation in gene expression patterns in breast tumors using complementary DNA microarrays has been reported.[33] It is already recognized the significant progress in identifying genomic profiles associated with risk of

distant metastasis and the probably association between these multi gene assays and the risk of local recurrence, and whether this gene signatures provide better predictions of clinical outcome than the traditional anatomical and pathological standards.[34,35] All these gene expression profiles have been used to determine the need for additional adjuvant treatment in breast cancer patients.[36]

Whether this gene expression based predictive indexes can be used to select patients for ALND is still under investigation. As far as now, the information provides by the gene expression profiles do not impact in the surgical management of the patients. Other clinical trials trying to identify patients with low molecular risk of relapse, derived from gene signatures, despite the number of positive axillary lymph nodes, is a subject of intense investigation.[37]

In conclusion, the TTL assessed intraoperatively by the whole SLN analysis by OSNA is a reproducible diagnostic technique for predicting additional non-SLN metastases. Furthermore, patients with higher copy number of CK19 mRNA, greater than 1.2×10^5 copies/μL and greater than 5×10^5 copies/μL in HR positive patients, had a higher frequency of non-SLN metastases. The improvement over frozen section is the advantage that the OSNA technique is a standardized and automated assay that can determine with no false negative the status of the SLNs intraoperatively.

Follow-up of patients who have had SLN assessed by OSNA and the TTL decision-making process will be required to identify the prognostic implications in term of locorregional recurrence and overall survival; this may lead to changes in the breast cancer staging system including the addition of the molecular intraoperative assay.

Conflict of interest statement

The authors have no actual or potential conflict of interest in relation to this article.

References

1. Lyman GH, Giuliano AE, Somerfield MR, et al. American Society of Clinical Oncology guideline recommendations for sentinel lymph node biopsy in early-stage breast cancer. *J Clin Oncol* 2005 Oct 20; **23**(30):7703–20.
2. Weaver DL, Ashikaga T, Krag DN, et al. Effect of occult metastases on survival in node-negative breast cancer. *N Engl J Med* 2011 Feb 3;**364**(5):412–21.
3. Fisher B, Bauer M, Wickerham DL, et al. Relation of number of positive axillary nodes to the prognosis of patients with primary breast cancer. An NSABP update. *Cancer* 1983 Nov 1;**52**(9):1551–7.
4. Abdessalam SF, Zervos EE, Prasad M, et al. Predictors of positive axillary lymph nodes after sentinel lymph node biopsy in breast cancer. *Am J Surg* 2001 Oct;**182**(4):316–20.
5. Albertini JJ, Lyman GH, Cox C, et al. Lymphatic mapping and sentinel node biopsy in the patient with breast cancer. *J Am Med Assoc* 1996 Dec 11;**276**(22):1818–22.
6. Jakub JW, Bryant K, Huebner M, et al. The number of axillary lymph nodes involved with metastatic breast cancer does not affect outcome as long as all disease is confined to the sentinel lymph nodes. *Ann Surg Oncol* 2011 Jan;**18**(1):86–93.
7. Houvenaeghel G, Nos C, Giard S, et al. A nomogram predictive of non-sentinel lymph node involvement in breast cancer patients with a sentinel lymph node micrometastasis. *Eur J Surg Oncol* 2009 Jul; **35**(7):690–5.
8. van la Parra RF, Peer PG, Ernst MF, et al. Meta-analysis of predictive factors for non-sentinel lymph node metastases in breast cancer patients with a positive SLN. *Eur J Surg Oncol* 2011 Apr;**37**(4):290–9.
9. Van Zee KJ, Manasseh DM, Bevilacqua JL, et al. A nomogram for predicting the likelihood of additional nodal metastases in breast cancer patients with a positive sentinel node biopsy. *Ann Surg Oncol* 2003 Dec;**10**(10):1140–51.
10. Edge SBBD, Compton CC, Fritz AG, et al. *AJCC cancer staging manual*. 7th ed. New York, NY: Springer; 2010.
11. Cserni G, Amendoeira I, Apostolikas N, et al. Pathological work-up of sentinel lymph nodes in breast cancer. Review of current data to be considered for the formulation of guidelines. *Eur J Cancer* 2003 Aug;**39**(12):1654–67.
12. Tsujimoto M, Nakabayashi K, Yoshidome K, et al. One-step nucleic acid amplification for intraoperative detection of lymph node metastasis in breast cancer patients. *Clin Cancer Res* 2007 Aug;**13**(16):4807–16.
13. Snook KL, Layer GT, Jackson PA, et al. Multicentre evaluation of intraoperative molecular analysis of sentinel lymph nodes in breast carcinoma. *Br J Surg* 2011 Apr;**98**(4):527–35.
14. Visser M, Jiwa M, Horstman A, et al. Intra-operative rapid diagnostic method based on CK19 mRNA expression for the detection of lymph node metastases in breast cancer. *Int J Cancer* 2008 Jun;**122**(11):2562–7.
15. Schem C, Maass N, Bauerschlag DO, et al. One-step nucleic acid amplification – a molecular method for the detection of lymph node metastases in breast cancer patients; results of the German study group. *Virchows Arch* 2009 Feb;**454**(2):203–10.
16. Tamaki Y, Akiyama F, Iwase T, et al. Molecular detection of lymph node metastases in breast cancer patients: results of a multicenter trial using the one-step nucleic acid amplification assay. *Clin Cancer Res* 2009 Apr;**15**(8):2879–84.
17. Feldman S, Krishnamurthy S, Gillanders W, et al. A novel automated assay for the rapid identification of metastatic breast carcinoma in sentinel lymph nodes. *Cancer* 2011 Jun;**117**(12):2599–607.
18. Rubio IT, Aznar F, Lirola J, et al. Intraoperative assessment of sentinel lymph nodes after neoadjuvant chemotherapy in patients with breast cancer. *Ann Surg Oncol* 2010 Jan;**17**(1):235–9.
19. Hammond ME, Hayes DF, Dowsett M, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. *Arch Pathol Lab Med* 2010 Jul; **134**(7):e48–72.
20. Dowsett M, Nielsen TO, A'Hern R, et al. Assessment of Ki67 in breast cancer: recommendations from the International Ki67 in Breast Cancer working group. *J Natl Cancer Inst* 2011 Nov 16;**103**(22):1656–64.
21. Cserni G. Intraoperative analysis of sentinel lymph nodes in breast cancer by one-step nucleic acid amplification. *J Clin Pathol* 2012 Mar;**65**(3):193–9.
22. Khaddage A, Berremila SA, Forest F, et al. Implementation of molecular intra-operative assessment of sentinel lymph node in breast cancer. *Anticancer Res* 2011 Feb;**31**(2):585–90.
23. Osako T, Iwase T, Kimura K, et al. Accurate staging of axillary lymph nodes from breast cancer patients using a novel molecular method. *Br J Cancer* 2011 Oct 11;**105**(8):1197–202.
24. Castellano I, Macri L, Deambrogio C, et al. Reliability of whole sentinel lymph node analysis by one-step nucleic acid amplification for intraoperative diagnosis of breast cancer metastases. *Ann Surg* 2012 Feb;**255**(2):334–42.
25. Osako T, Iwase T, Kimura K, et al. Intraoperative molecular assay for sentinel lymph node metastases in early stage breast cancer: a comparative analysis between one-step nucleic acid amplification whole node

assay and routine frozen section histology. *Cancer* 2011 Oct 1; **117**(19):4365–74.
26. Tamaki Y, Sato N, Homma K, et al. Routine clinical use of the one-step nucleic acid amplification assay for detection of sentinel lymph node metastases in breast cancer patients: results of a multicenter study in Japan. *Cancer* 2012 Jul 15;**118**(14):3477–83.
27. Ohi Y, Umekita Y, Sagara Y, et al. Whole sentinel lymph node analysis by a molecular assay predicts axillary node status in breast cancer. *Br J Cancer* 2012 Oct 9;**107**(8):1239–43.
28. Mittendorf EA, Hunt KK, Boughey JC, et al. Incorporation of sentinel lymph node metastasis size into a nomogram predicting nonsentinel lymph node involvement in breast cancer patients with a positive sentinel lymph node. *Ann Surg* 2012 Jan;**255**(1):109–15.
29. Meretoja TJ, Leidenius MH, Heikkila PS, et al. International multicenter tool to predict the risk of nonsentinel node metastases in breast cancer. *J Natl Cancer Inst* 2012 Dec 19;**104**(24):1888–96.
30. Giuliano AE, Hunt KK, Ballman KV, et al. Axillary dissection vs no axillary dissection in women with invasive breast cancer and sentinel node metastasis: a randomized clinical trial. *JAm Med Assoc* 2011 Feb 9;**305**(6):569–75.
31. Caudle AS, Hunt KK, Tucker SL, et al. American College of Surgeons Oncology Group (ACOSOG) Z0011: impact on surgeon practice patterns. *Ann Surg Oncol* 2012 Oct;**19**(10):3144–51.
32. Cserni G, Gregori D, Merletti F, et al. Meta-analysis of non-sentinel node metastases associated with micrometastatic sentinel nodes in breast cancer. *Br J Surg* 2004 Oct;**91**(10):1245–52.
33. Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. *Nature* 2000 Aug 17;**406**(6797):747–52.
34. Mamounas EP, Tang G, Fisher B, et al. Association between the 21-gene recurrence score assay and risk of locoregional recurrence in node-negative, estrogen receptor-positive breast cancer: results from NSABP B-14 and NSABP B-20. *J Clin Oncol* 2010 Apr 1;**28**(10): 1677–83.
35. Rutgers E, Piccart-Gebhart MJ, Bogaerts J, et al. The EORTC 10041/ BIG 03-04 MINDACT trial is feasible: results of the pilot phase. *Eur J Cancer* 2011 Dec;**47**(18):2742–9.
36. Oliveira M, Cortes J, Bellet M, et al. Management of the axilla in early breast cancer patients in the genomic era. *Ann Oncol* 2012 Nov 19; http://dx.doi.org/10.1093/annonc/mds592.
37. Gonzalez-Angulo AM, Barlow WE, Gralow J, et al. SWOG S1007: a phase III, randomized clinical trial of standard adjuvant endocrine therapy with or without chemotherapy in patients with one to three positive nodes, hormone receptor (HR)-positive, and HER2-negative breast cancer with recurrence score (RS) of 25 or less. *J Clin Oncol* 2011;**29**. [suppl; abstr TPS104].