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CLINICAL TRIAL

Sentinel lymph node analysis in breast cancer: contribution of one-step nucleic acid amplification (OSNA)

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Abstract One-step nucleic acid amplification (OSNA, Sysmex, Kobe, Japan) offers an excellent opportunity for accurate exhaustive sentinel lymph node (SLN) examination in breast cancer patients. Calibrated with conventional postoperative histology, this molecular technique yields comparable results intraoperatively, expressed as micrometastasis, macrometastasis or no metastasis depending on the CK19 mRNA copy number amplified in SLN lysates. We applied OSNA to detect metastasis in 810 SLNs from 367 patients with early stage breast cancer. We compared the rate of OSNA-positive SLNs in patients with invasive breast cancer (< 2 cm) versus the rate observed in a historical cohort using conventional histological examination of SLNs. No significant difference was observed, the OSNA assay was positive in 24.4% of patients, compared with positive histology in 24.8% in the historical cohort if including patients with isolated tumour cell (ITC) and in 23.4% excluding them. Opportunities for optimised patient management using OSNA are discussed: intraoperative detection of OSNA-positive SLNs enables axillary lymph node dissection (ALND) during the same procedure; standard OSNA techniques enable the establishment of homogeneous groups based on examination of whole SLNs for valid comparisons between different centres.

Keywords Breast cancer · Sentinel lymph node biopsy · Molecular analysis · Axillary lymph node dissection

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Introduction

Sentinel lymph node (SLN) biopsy is a highly accurate predictor of overall axillary status used in patients with clinically lymph node-negative early stage breast carcinoma. SLN biopsy safely avoids axillary lymph node (ALN) biopsy when the SLN is disease-free, reducing arm morbidity and improving quality of life compared with axillary lymph node dissection (ALND) [1]. Protocols for SLN biopsy evaluation vary widely between healthcare centers; some perform an intraoperative examination of the SLN, others do not because of the low sensitivity of intraoperative pathological techniques. Intraoperative SLN assessment has the obvious advantage of allowing immediate conversion to ALND, thereby avoiding the morbidity, inconvenience and cost of a second operation. Pathological techniques for intraoperative SLN analysis include frozen section and touch imprint cytology. Sensitivity of touch imprint cytology is limited by the small amount of tissue investigated, about 50% in the presence of macrometastasis (>2 mm) and 10% for micrometastasis (0.2–2 mm) [2]. Sensitivity is somewhat better with frozen section analysis, but at the cost of tissue loss for paraffin blocks postoperative histopathology [3, 4]. In contrast, the molecular techniques allow intraoperative examination of the entire SLN with the same sensitivity as conventional gold-standard postoperative histopathology. The one-step nucleic acid amplification (OSNA) assay

(Sysmex, Kobe, Japan) is commercially available (Sysmex, Kobe, Japan) for clinical use. The whole OSNA process is CE approved: SLN lysate preparation reagents, the automated RD100i system for reagent and sample distribution as well as molecular amplification. The principle of the test is to detect amplification of cytokeratin 19 (CK19) mRNA, which is not normally found in lymph nodes except in the presence of metastases, by reverse-transcription loop-mediated isothermal amplification (RT-LAMP) [5]. The OSNA assay yields semi-quantitative results and can detect nodal metastasis measuring > 0.2 mm at rates comparable to conventional pathological examination with an overall concordance of 98.2% [6]. Validation studies [7–11] confirmed that OSNA can be used intraoperatively to detect sentinel node involvement with a sensitivity of 91.7–100% and a specificity of 90.8–97.1%. OSNA has been adopted in our institution as an intraoperative test for the detection of SLN metastasis and management of patients with breast cancer. The purpose of this study is to present our first results with OSNA assays performed in a routine clinical setting in 365 patients with invasive and in situ breast cancer. Test results are subgrouped by histological characteristics of the breast tumour and also compared with conventional gold-standard histopathology results for SLN biopsies in a historical cohort in our institution [2].

Patients and methods

Patients

From October 2008 to June 2010, 367 patients (total study population) with clinically node-negative early stage breast cancer underwent an axillary SLN procedure in the Comprehensive Cancer Center E Marquis in Rennes, France. SLN biopsy was performed in patients with breast tumour size less than T2, unifocal breast cancer, and no homolateral breast surgery in the past. A few patients with pT2 were included because the size of the tumour was underestimated before surgery. The SLN was assessed with OSNA intraoperatively to determine the management strategy. ALND was performed during the same surgery when the SLN was OSNA-positive. Patient characteristics are presented in Table 1.

The OSNA results were compared with conventional postoperative histopathology results from an historical cohort of patients who had undergone an SLN biopsy procedure in the same institution one year earlier (Table 1). Detailed results from the historical cohort have been published elsewhere [2]. Patients with in situ carcinoma (43 patients), tumour size $>$ T1c (50 patients) or invasive carcinoma other than ductal or lobular forms (15 patients

Table 1 Patients demographics and tumour characteristics

		OSNA total Cohort		OSNA Cohort for comparison to historical cohort	Historical Cohort
Number of patients		367		258	355
Medium age of patients		56.8 years		56.8	56.9
Tumour histology	Ductal invasive carcinoma	248		212	313
	Lobular invasive carcinoma	60	→	46	42
	In situ carcinoma	43			
	Other carcinoma	16			
Tumour size	1a	21		19	16
	1b	104		93	125
	1c	148		146	214
	2	50			
	Missing values	44			
SBR Grade	1	94			
	2	171			
	3	68			
	Missing values	34			
Hormones receptor status	Estrogen/progesterone +	300			
	Estrogen/progesterone −	51			
	HER2 +	21			
	Triple Negative	29			

classified other in Table 1) were excluded from the OSNA cohort for result comparison between OSNA and histology use, to avoid any bias because these categories of patients were not represented in the historical cohort. SLN status was then finally compared between 258 patients in the OSNA cohort and 355 patients from the historical cohort (Fig. 1).

Sentinel lymph node sampling method

The localisation of the sentinel node was identified using the combined method: ^{99m}technetium-labelled colloid (Nanocoll®, Amersham Swan, Eindhoven, the Netherlands) injected the day before surgery and 3 h after axillary lymphoscintigraphy, then, on the day of the procedure, subcutaneous injection of 2 ml of patent blue dye (Guerbet® Patent Blue V, Guerbet Laboratory, Aulnay-sous-Bois, France). SLNs were cut by the pathologist and touch imprints were performed intraoperatively. A 1 mm thick central slice was stained for postoperative histology. The remaining portion of the node was used for OSNA analysis intraoperatively.

In contrast with the validation studies, in the present study OSNA was performed on nearly the entire SLN to reduce the likelihood of detecting a positive reaction only in the part reserved for standard postoperative histology (and subsequently a second procedure for ALND).

The final result of the histological examination of the central slice was not compared with the OSNA result because of the tissue allocation bias created by using most of the material for the molecular method.

OSNA Assay

The OSNA assay has been described in detail in a previous report [6]. In brief, after removing extranodal tissue and lipid, the SLN is homogenised and shortly centrifuged according to the manufacturer's instructions (Sysmex, Kobe, Japan). SLNs weighing more than 600 mg have to be cut and analysed separately with two or more molecular analyses. OSNA analysis is carried out in duplicate with a pure and a diluted sample (1/10) of SLN lysates without prior isolation and purification of mRNA. After a 16 min amplification time, the CK19 mRNA copy number per µl of lysate determined the node status defined as follows: copy number <250 = no metastasis, copy number 250–5000 = micrometastasis and copy number >5000 = macrometastasis [6]. The OSNA assay discriminated macrometastasis from micrometastasis well but was not calibrated to detect isolated tumour cells

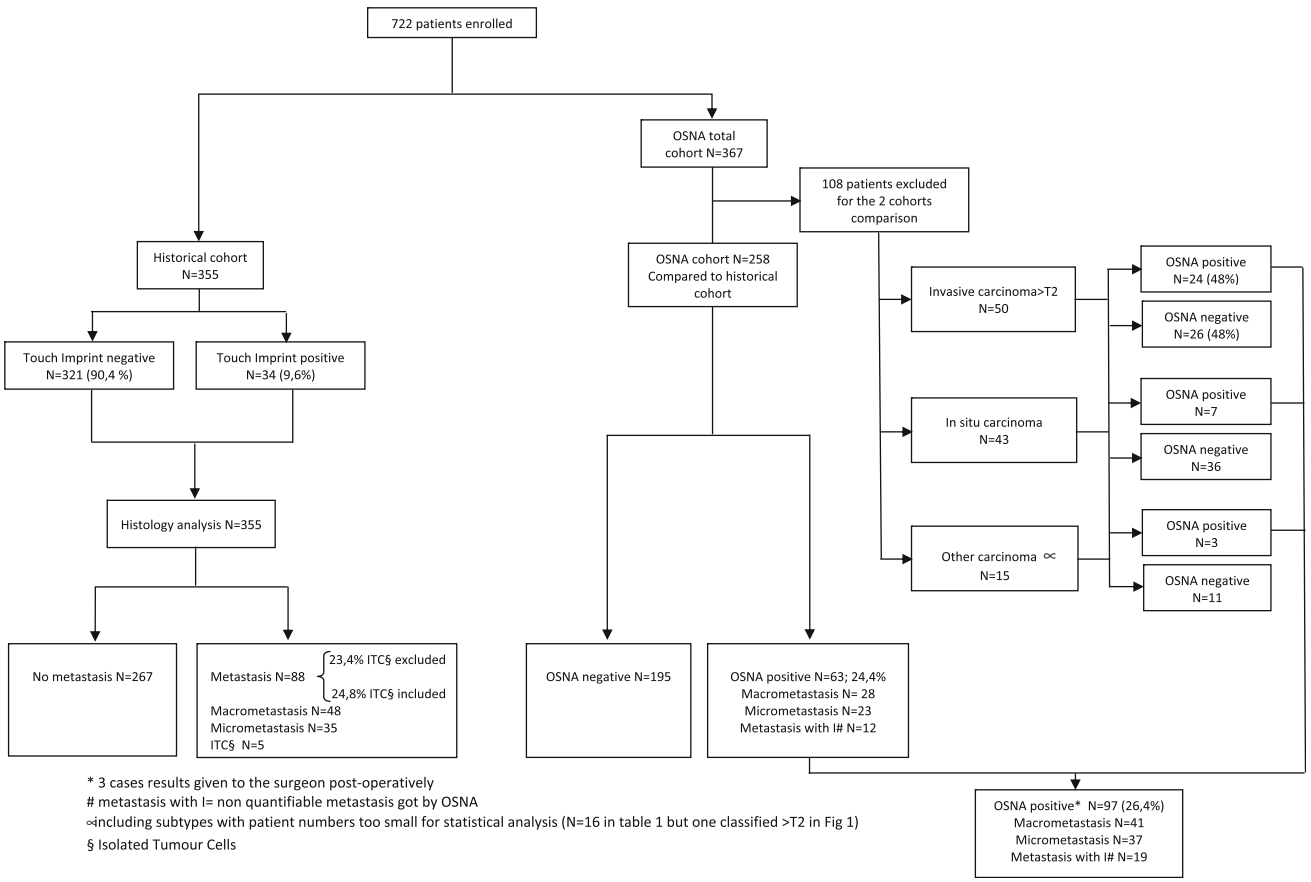

Fig. 1 Flow chart for the comparison of OSNA and historical cohorts

(ITC) [6]. If copy numbers are >250 in the diluted preparation only, the OSNA result is designated as positive with inhibition of the amplification reaction; the SLN metastasis cannot be semi-quantified because of potential interference with the molecular detection.

In our study, patients with at least one SLN macrometastasis were classed as macrometastatic, those with at least one SLN micrometastasis as micrometastic, and those with at least one metastasis with inhibition as metastatic.

Histological evaluation

The intraoperative examination consisted of a cytological examination of the touch imprinted sections. In the OSNA population, the SLN was sectioned to keep a 1 mm thick section for final histology and in the historical cohort the SLN was sectioned every 2 mm. The resulting sections were directly touch imprinted on the glass slide. The slides were then room-dried and stained with Toluidine blue and analysed under the microscope. The thick sections were then put into a cassette and fixed in formol for the final paraffin-embedded histological examination. In the event of metastatic cell clusters being identified on the cytology slide, the surgeon then carried out complete axillary nodal dissection during the same surgery. The final histological examination consisted of a detailed analysis of the SLN tissue sections embedded in paraffin blocks, and sectioned every 250 μm until the block was completely cut. Each level was initially stained with standard H&E. If no metastasis were revealed by conventional staining, then immunohistochemical (IHC) labelling was carried out using an anti-pancytokeratin antibody (AE1/AE3 clones, Dako, Trappes, France): the SLN was examined by IHC labelling of all levels.

Final examination of axillary non-SLNs was investigated by permanent histology (each 2 mm section of the lymph node was analysed with H&E staining) in both the OSNA and historical cohort.

Statistical analyses

Statistical analyses were performed using SAS 9.1 software (Statistical Analysis System, Cary, NC). Association between parameters was assessed using the chi-2 test or Fisher's exact test (for $n < 5$); P values < 0.05 were considered statistically significant.

Results

SLNs in the OSNA cohort ($n = 367$)

SLN status was determined with OSNA assay in 367 patients. Altogether, 810 SLNs were investigated, with 2.2 SLNs/patient on the average. Cutting SLNs weighing more than 600 mg led to 901 amplification runs.

The time required for the final results of the OSNA assay (including transport to the laboratory) depended on the number of SLNs studied. The mean time (± SD) was 32.9 ± 4.9 min for 1 SLN ($n = 94$), 36.4 ± 4.5 min for 2 SLNs ($n = 144$), 41.6 ± 5.2 min for 3 SLNs ($n = 87$), 48.5 ± 8.7 min for 4 SLNs ($n = 39$).

At least one SLN was OSNA-positive in 97 patients (26.43%): 41 patients were classified as macrometastatic, 37 as micrometastatic and 19 as metastatic with inhibition of the amplification reaction. The standard histology examination identified tumour cells in the central section in 57 patients (15.53%).

Invasive carcinoma patients in the OSNA cohort ($n = 324$)

ALND was performed during the same surgery as the SLN biopsy in 91 patients, following intraoperative OSNA detection of SLN metastasis in 90/324 (27.7%) patients. In one OSNA negative patient ALND was performed according to the surgeon's decision. A second procedure was performed for ALND in 12 patients. In three patients this was because of technical problems with intraoperative OSNA use; in nine patients a few tumour cells or small micrometastases in the central slice were found with final histology. All nodes removed during the ALND in these nine patients were negative. Metastatic non-SLNs were found in 34.4% of the patients classed as macrometastatic by OSNA, in 10.8% of those classed micrometastatic, and in 5.2% of those classed metastatic with inhibition.

The results were grouped by histological subtype in Table 2: ductal carcinoma (248 patients), lobular carcinoma (60 patients) and others (16 patients, including subtypes with patient numbers too small for statistical analysis). There was no significant difference in OSNA positivity between patients with ductal carcinoma (27.8%) and lobular carcinoma (30%). Correlation between SLN OSNA-macrometastasis and ALND metastasis was significant in both groups. SLN OSNA-micrometastasis was significantly correlated with ALND metastasis for ductal carcinoma, but not for lobular carcinoma.

SLN OSNA results and classical breast tumour prognostic factors (Table 3) ($n = 367$ patients)

A positive OSNA assay was significantly correlated with breast tumour size: in 14.3% of T1a tumours and 48% of T2 tumours a positive assay was found. OSNA was positive in 39.3% of patients with Ki67 ≥ 20% tumours and 25.2% in those with Ki67 < 20% tumours. The difference was significant. OSNA assay was positive for 47.6% of

Table 2 Lymph nodes with metastasis in ALND if SLN positive with OSNA

	% of patients SLN + OSNA	OSNA result patient SLN +	% Patients with lymph node metastasis in ALND	Correlation between lymph node metastasis in ALND and SLN + OSNA
Ductal invasive carcinoma	27.8% (69/248)	30 Macrometastasis	33.3% (10/30)	$P < 0.0001$
		26 Micrometastasis	11.5% (3/26)	$P = 0.0129$
		13 Metastasis with inhibition	7.6% (1/13)	*NS*
Lobular invasive carcinoma	30.0% (18/60)	10 Macrometastasis	40% (4/10)	$P = 0.002$
		6 Micrometastasis	16.6% (1/6)	
		2 Metastasis with inhibition	(0/2)	

HER2-positive patients and 26.2% for HER2-negative patients. The difference was again significant. Conversely, univariate analysis showed that a positive OSNA result was not significantly correlated with patient age, breast tumour hormone status or Scarff-Bloom-Richardson (SBR) tumour grade. Similarly, OSNA positivity was not significantly different between patients with triple negative versus non triple negative breast tumours.

Table 3 Univariate analysis for OSNA SLN involvement according to breast primary tumour prognostic factors

	N	% Patients SLN + OSNA	*P* value
Age			
< 40	25	40	*NS*
40–50	87	26.4	
50–60	107	21.5	
60–70	108	26.9	
> 70	40	30	
Size of primary tumour			
T1a	21	14.3	0,0009
T1b	102	18.6	
T1c	148	29	
T2	50	48	
Hormone receptor Status			
Estrogen/progesterone +	300	27.7	*NS**
Estrogen/progesterone −	51	25.5	
Bloom-Richardson grade			
1	94	22.3	*NS**
2	171	27.5	
3	68	35.3	
Ki67 < 20%	135	25.2	0.05
Ki67 ≥ 20%	56	39.3	
HER2 +	21	47.6	0.033
HER2 −	302	26.2	
Triple negative			
Yes	29	20.7	*NS*
No	310	28.1	

NS non significative

Immunohistochemistry (IHC) for Ki67 semi quantification (M7240 clone, Dako, Trappes, France)

IHC for HER2 determination (A0485, Dako, Trappes, France) if positive ++ completed by FISH (Fluorescent In SituHybridization) to determine final status

OSNA results (258 patients) versus standard histology in the historical cohort (355 patients)

The historical cohort included 355 patients with invasive ductal or lobular carcinoma measuring < 2 cm. The OSNA cohort was thus restricted to patients with stage T1a, T1b or T1c ductal or lobular carcinoma. This gave 258 patients whose intraoperative OSNA results were compared with the final histology results from the historical cohort (Fig. 1). Two rates were determined for histology positivity, either including or excluding the presence of isolated tumour cells (ITC). The OSNA assay was positive in 24.4% (63/258) of patients, compared with positive histology in 24.8% (88/355) if including the 5 patients with ITC and in 23.4% excluding them. These rates were not significantly different. OSNA assay detected more positive nodes in patients with a small tumour (T1a) (Fig. 2), but comparison of the OSNA cohort and the historical cohort showed no significant difference by tumour subtype (Fig. 3) or size.

SLN status in the 63 patients with an OSNA-positive SLN (63/258 = 24.4%) led to the following classification: macrometastatic ($n = 28$, 45%), micrometastatic ($n = 23$, 36.5%) and metastatic with inhibition ($n = 12$, 19%).

In the historical cohort, there were 88 patients with a metastatic SLN (88/355 = 24.8%) who were classed macrometastatic ($n = 48$, 54.5%), micrometastatic ($n = 35$, 39.8%) and 'ITC only' ($n = 5$, 5.7%).

Discussion

Intraoperative SLN result, the prime advantage of OSNA

Having reliable results in the operative theatre is a major advantage for patients: a second procedure for ALND can

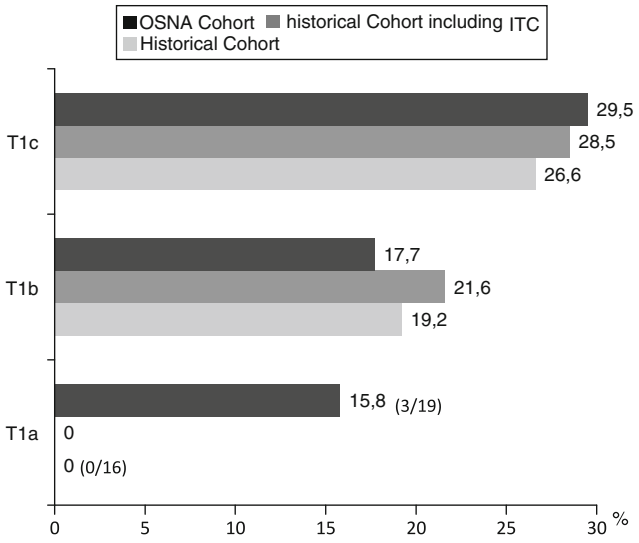

Fig. 2 Univariate analysis between % SLN involvement in OSNA restricted cohort vs. % SLN involvement in Historical cohort by tumour size

be avoided and adjuvant therapy can be initiated rapidly if necessary.

In this observational study considering early stage invasive breast cancer (< 2 cm), intraoperative OSNA identified positive SLNs in 24.4% of patients, a rate very similar to the 23.4% recorded by gold-standard postoperative histopathology in the same institution before implementation of the new molecular technique. Furthermore, OSNA detected more positive SLNs of small tumours (T1a) than conventional histopathology (15.8% vs. 0%, NS) and identified SLN metastasis well in high-risk patients (HER2 +, Ki67 > 20%, triple negative).

The semi-quantitative OSNA classification was also well correlated with non-sentinel node involvement: 34.4% of OSNA-macrometastasis patients had axillary lymph node metastasis compared with only 12.1% of the OSNA-micrometastasis patients. According to data in the literature, the risk of axillary lymph node metastasis is about 40% in the presence of SLN macrometastasis [12, 13] and about 14% for SLN micrometastasis [14], 20% for 1–2 mm and 10% for 0.2–1 mm micrometastasis [13]. Our rates are in general agreement, though slightly lower, especially for SLN macrometastasis where the previous touch imprint may have screened out some large SLN macrometastasis patients known to have a high risk of axillary lymph node involvement.

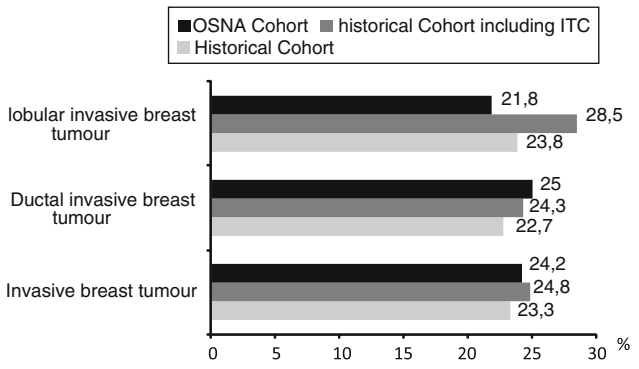

Fig. 3 Univariate analysis between %SLN involvement in OSNA restricted cohort vs. %SLN involvement in historical cohort by tumour subtype

Standard SLN assessment, a second advantage

Since the OSNA assay is standardised it produces comparable results from different institutions; patients can be identified as SLN OSNA-negative, OSNA-micrometastasis, or OSNA-macrometastasis. This is an important advantage over postoperative histopathology where reproducible identification of micrometastasis patients is particularly difficult since it is rather impractical to mount, stain and microscopically examine every section through the SLN paraffin blocks [15]. With the molecular technique, the whole node can be analysed rapidly, providing reproducible results, especially for micrometastasis and probably also for ITC spreading through the node. The issue of ITC requires special attention since the OSNA assay has been designed [7] only to detect tumour deposits measuring at least 0.2 mm^3, not ITC. We found good agreement between OSNA assay and histology when ITCs were included, suggesting the OSNA assay can detect both tumour cells organised in micrometastasis and ITCs, giving a semi-quantitative assessment of tumour volume in the whole node. What has to be done now is to consider OSNA results in light of the clinical paradigm. How are percentages of positive ALN correlated with different prognostic groups of breast cancer patients as defined by amplified CK19 mRNA copy numbers? Can OSNA discriminate patients with different disease-free survivals? To date, clinical studies have compared macrometastasis versus micrometastasis as determined by histology. Perhaps OSNA metastasis quantification of the whole SLN could provide more clinically pertinent cut-off levels expressed clearly by CK19 mRNA copy numbers.

Open questions about OSNA

Is whole node lysate a valid option for OSNA analysis?

Considering the good concordance between histology and OSNA, SLNs would no longer have to be cut to reserve a small portion for final standard histopathology; this could create tissue allocation bias with difficulty for SLN involvement interpretation. The disadvantage is that no

tissue would be left to search for lymph node pathology other than breast metastasis; it could be suggested to keep imprint colouration of SLN. SLN lysates stored at −70°C would be sufficient for subsequent molecular analyses after mRNA extraction (it is known that mRNA could be stored at −70°c several years), SLN lysate could be re-analysed within one month after the initial OSNA run suggesting a mRNA extraction on the lysate for long-term storage.

If however the whole node is homogenised for OSNA analysis, the primary tumour might have to be tested for CK19 expression to rule out false negative OSNA assay because of low CK19 expression? CK19 protein expression has been found in 98% of breast cancers [16] and a recent study [17] examining 116 cases of special histological types of invasive breast carcinoma (micropapillary, mucinous, apocrine, tubular, medullary and mixed breast carcinoma) reported that CK19 immunoreactivity in tissue microarray increased to 100% after repeating the immunostain in whole tissue because of focal expression; the authors conclude that OSNA assay could be used in all breast cancer cases. Furthermore, all clinical studies have found good agreement between OSNA and histopathology. OSNA did detect CK19 mRNA even if no CK19 protein expression was found in IHC [7].

How to quantify OSNA metastasis with amplification inhibition?

OSNA detected metastatic SLNs in 97 patients, including 19 classed OSNA-positive with inhibition of the CK19 mRNA amplification. In these 19 patients, metastasis quantification was impossible because of interference in the molecular reaction. This absence of SLN quantification can be a real problem, as illustrated in recent studies demonstrating the controversial meaning of micrometastasis [18, 19]. The mechanisms of interference which generate inhibition are unknown; an evaluation is ongoing. At the present time, the only solution is to quantify metastasis with inhibition using other molecular investigations, after mRNA extraction and purification, to eliminate interferences. Cases which could not be classified as macrometastasis or micrometastasis might be excluded from immediate ALD if SLN micrometastasis is not always an indication of ALND.

Nevertheless, Sysmex is developing a new software version to distinguish micrometastic from macrometastasis tumour load in samples with inhibited amplification.

Surgery organisation with intraoperative OSNA

In our practise all the OSNA results were communicated intraoperatively to the surgeon except three cases because of technical problem with OSNA assay. OSNA results for one SLN can be obtained in half an hour, with five minutes more for each supplementary SLN. This delay must be considered for organising surgery, most of the time the surgeon has to wait for the OSNA result and we suggest that if it's impossible to wait, the OSNA result will be considered postoperatively, similar to final histological examination when it was in use. In these cases the advantage of OSNA intraoperative result is lost but the other advantages of OSNA standardization and whole node examination remain.

Cost analysis of the implementation of breast cancer sentinel node intraoperative molecular diagnosis

A detailed cost analysis has not yet been finalised in our institution, but two studies, the first with Genesearch BLN assay (Veridex, LLC Warren, NJ) [20], the second with OSNA assay [21] achieved a saving of money to the hospital with implementation of breast cancer SLN intraoperative molecular diagnosis, because saving of theatre time and hospital bed utilisation.

OSNA in the future

OSNA assay has been standardised in agreement with conventional histopathology, allowing well-defined discrimination between SLN micrometastasis and SLN macrometastasis. Our results, like those from other users of OSNA illustrate this very good correlation with histopathology results. The question now becomes how to better exploit this OSNA information in a clinically pertinent manner. An important advantage of OSNA is its accuracy. A nomogram project is already initiated to draw on this accuracy in different breast cancer populations. This nomogram using CK19 mRNA copy number in SLN, other classical histological factors, and molecular characteristics of the breast tumour would like to predict the risk of involvement of other ALNs and clinical outcome. This would optimise OSNA assay yield in terms of answering unresolved clinical questions. What is the correlation between breast cancer survival and tumour burden in ALNs? What is the risk of ALN involvement in patients with a metastatic SLN and the pertinence of ALND? There is some controversy, particularly since it is now known that the risk of axillary recurrence is incredibly low (< 1%), as shown in the recent Z0011 study [22]. Certain authors have noted that when metastatic SLN patients are treated with breast conservation and systemic therapy, ALND does not improve 5-yr survival rates over SLN dissection alone [23]. The authors recalled, however, that a 19% rate of axillary failure was observed in the older study B04, the difference between the two studies being the treatment. New protocols, together with better comprehension and evaluation of

breast cancer have thus contributed to optimise breast cancer treatment. Adding the accuracy of the OSNA assay should enable even more precise evidence-based decision-making for breast cancer patients with SLN involvement.

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