Implementation of Molecular Intra-operative Assessment of Sentinel Lymph Node in Breast Cancer

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Abstract. *Background: Sentinel lymph node (SLN) biopsy is used as a staging procedure in early breast cancer, however, histology based intra-operative assessment of the SLN status has a low sensitivity. The one-step nucleic acid amplification (OSNA) method was developed to detect metastases by amplification of cytokeratin (CK) 19 mRNA. Experience with OSNA during a French multi-centric prospective study, as well as intra-operative clinical routine use, is reported. Patients and Methods: For the clinical study 80 SLNs from 46 patients were assessed. During routine use, the central slice of the SLN from 197 patients was investigated by permanent histology and the remainder was assessed by OSNA. Results: During the clinical study, OSNA detected 15/17 metastases, including all the macrometastases, reaching a 96.3% concordance rate, 88.2% sensitivity and 98.4% specificity. During routine use, both OSNA and histology detected 25 patients with metastasis. Conclusion: OSNA is an accurate tool for intra-operative assessment of SLN status and could reduce the need for second surgery.*

The sentinel lymph node (SLN) is the first lymph node receiving lymphatic drainage from the primary tumour and is highly predictive of the status of the axillary lymph nodes in women afflicted with breast cancer (1). SLN biopsy as part of the staging procedure has greatly improved the management of clinically node-negative breast cancer patients (2), and its use has reduced the morbidity associated with axillary lymph node dissection (ALND) (3). Conventional procedures for intra-operative assessment such as frozen slides and imprint cytology have low sensitivity, from 57 to 74% as recently reported (4) and lead to second surgeries for 10 to 17% of patients (5) when a metastatic SLN is found with post-operative permanent histology (6, 7).

With histology performed on paraffin-embedded SLN tissue after the primary surgery, upstaging rates in breast cancer patients between 9-47% have been reported (8). Consistent interpretation of these data is difficult since they are based on a variety of histological protocols, including step sectioning and immunohistochemistry (IHC), as well as variable interpretations of small tumour deposits (9). In addition, it has recently been illustrated that by slicing the SLN into 2 mm tissue blocks and preparing one or more levels of haematoxylin and eosin (H&E) staining from each block, as recommended by several international guidelines, metastases smaller than 2 mm might be missed (10). If these non-detected metastatic foci represent the only tumour deposits in the SLN the patient will be falsely classified as pN0. In addition, the maximum size of the metastasis might be underestimated because of the 2-dimensional view presented by histological sections (10). Thus, a standardised intra-operative technique which investigates most of the SLN, thereby avoiding sampling errors, is needed.

Several molecular assays applied for the assessment of the SLN status have shown higher positivity rates when compared to routine H&E staining as well as IHC (11, 12), but the intra-operative use of these approaches is hampered by a prolonged operation time including purification of RNA. Two commercial intra-operative molecular techniques aiming to avoid second axillary dissection have been investigated (13, 14). These techniques reach the highest level of sensitivity compared to touch imprint or frozen sections. The first kit based on quantitative RT-PCR did not distinguish micrometastases from macrometastases (15) and is no longer available. This could create a problem in clinics where ALND is exclusively employed upon detection of macrometastases and where patients with only micro-metastases are spared from further treatment (16). The one-step nucleic acid amplification (OSNA) method was developed to accurately detect metastases (≥0.2 mm) by rapid amplification of cytokeratin (CK) 19 mRNA in lymph nodes of breast cancer

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patients providing results within 30-40 minutes. The excellent performance of OSNA in comparison to extensive histology has been shown in four clinical studies (14, 17-19).

In the past, no intra-operative analysis of the SLNs was performed in the St. Etienne University Hospital because of the inherent false negative rate, particularly for lobular carcinoma and micrometastases, and because it is arduous and time consuming for pathologists. As a consequence, patients had to wait 7 to 14 days for the final judgement on the histological status of the SLNs, and when positive had to return for a second operation, which was both psychologically and physically very stressful for the patient, as well as cost-intensive for the hospital. The evaluation of a molecular assay performed during the primary surgery, but with the accuracy of permanent multi-level histology, was therefore attractive.

We first participated in a French multicentre prospective study (manuscript in preparation) to evaluate the intra-operative diagnostic performance of OSNA in comparison to post-operative extensive histological examination and then introduced this technique into our routine practice. Here, the experience of our centre in using OSNA during the clinical validation study and first routine use is reported.

Patients and Methods

Patients. Forty-six breast cancer patients from our centre were enrolled as part of the multicentric prospective clinical study from September to December 2007. The study was approved by the Comité de Protection des Personnes Ile de France II. One hundred and ninety-seven patients were registered for OSNA routine use.

For both patient cohorts (Table I) inclusion criteria were a minimum age of 18 years and assignment for SLN biopsy. Exclusion criteria were neoadjuvant treatment and the presence of metastatic disease other than breast carcinoma. All patients gave written consent prior to the surgical intervention.

Sentinel lymph node processing. During the clinical study, nodes were defatted after SLN biopsy and intra-operatively cut into four equal slices (a, b, c, d) of 1 to 2 mm thickness (Figure 1). Two alternate slices were analysed by OSNA (a and c), slices b and d were subjected to histology. The results of OSNA were not known to the investigator of histology and *vice versa*.

During this validation phase, in cases of discordant results, the lysates of the samples were subjected to additional molecular analyses (discordant case investigation, DCI) in order to detect whether the discordances were caused by tissue allocation bias (TAB), meaning that a metastasis was either located in the slices used for OSNA or the slices used for histology. In cases of TAB, the patients were excluded because the two methods could not be compared.

Concordance, sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) were calculated on the basis of the results obtained by this method comparison.

During routine use, a central 1 mm slice was assigned to histological work-up. The other two parts of the node were completely analysed by OSNA (Figure 1). Since the SLN tissue was unevenly allocated to histology and OSNA, statistical calculations for method comparison were not performed.

Table I. *Clinicopathological features of patients*.

	Clinical study (46)	Total	Routine use (197)	Total
Primary tumour status		46		197
T0	7		1	
Tis			21	
T1	34		141	
T2	2		30	
T3	2		1	
T4	1			
Tx			3	
Nodal status		46		197
pN0	43		162	
pN1	3		33	
pN2			2	
Histopathological type		46		197
Invasive ductal carcinoma	36		148	
Invasive lobular carcinoma	5		16	
Other invasive type			8	
Carcinoma *in situ*	5		21	
Not assessable			4	
Histological tumour grade		46		197
1	8		51	
2	21		90	
3	12		28	
In situ high grade	4		20	
In situ low grade	1		1	
Not assessable			7	

Histological examination. For the clinical study, slices b and d were embedded in paraffin and post-operatively cut at 200 μm intervals (5 levels, Figure 1). Each level was subjected to H&E and IHC staining for CK19 protein (Clone RCK108, Dako; Glostrup, Denmark) as well as IHC with AE1/AE3 (Clones AE1/AE3, Dako; Glostrup, Denmark) as a pan-cytokeratin marker. For routine use, a central slide of 1 mm from each SLN was analysed by 1 level of H&E staining and 1 level IHC (AE1/AE3). Non-SLNs (NSLN) were cut into 2 mm slices and 1 level of H&E staining was performed for each slice.

Tumour deposits were classified according to the TNM classification of the Union for International Cancer Control (UICC 6th edition) and the American Joint Committee on Cancer (AJCC 6th edition) (20, 21). The presence of a macrometastasis or micrometastasis was recorded as a positive histological result, isolated tumour cells (ITC), or a tumour-free SLN as a negative histological result.

One-step nucleic acid amplification (OSNA). OSNA was performed according to the manufacturer's instructions (Sysmex, Kobe, Japan) and as described recently (14, 17-19). The SLN slices were homogenised in 4 ml homogenising buffer Lynorhag (Sysmex). Afterwards, the homogenate was briefly centrifuged (Figure 1) and directly used as a template for reverse transcription loop-mediated isothermal amplification (RT-LAMP) (22). Amplification of *CK19* mRNA was automatically performed in an RD-100i instrument (Sysmex) with a ready-to-use reagent kit Lynoamp (Sysmex)

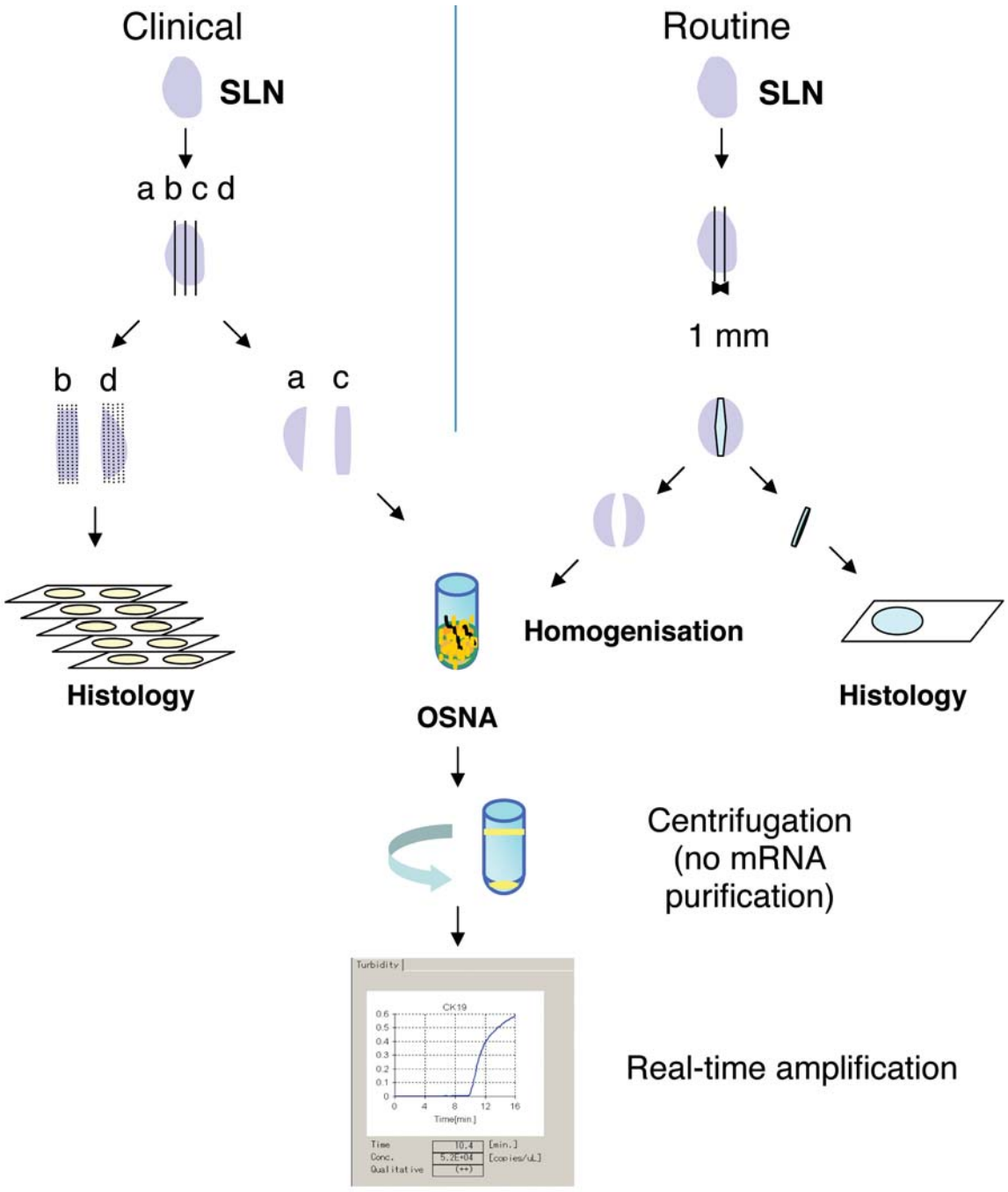

Figure 1. *Sentinel lymph node sampling procedure*.

consisting of a primer-nucleotide-mix, enzymes and *CK19* mRNA calibrators as well as positive and negative controls.

Prior to the sample run three different calibrators with defined *CK19* mRNA copy concentrations were used to establish a standard curve on the RD-100i. All the results were presented on the RD-100i in qualitative categories (++, +, –) and further specified by *CK19* mRNA copy number/μl: 0-249 copies (–), 250-5000 copies (+), and copy number >5000 (++). A result indicating a (+) was comparable to the presence of a micrometastasis and (++) to a macrometastasis (14).

Discordant case investigation (DCI). DCI consisted of quantitative reverse-transcriptase polymerase chain reaction (QRT-PCR) for *CK19* mRNA and two breast cancer-specific markers (SAM pointed

Table II. *OSNA versus histology: results of the clinical study (46 patients).*

		Histology (5 levels)			
		Macro-metastases	Micro-metastases	Negative	All
Per node					
OSNA	++	11	2	0	13
	+	2	0	1	3
	–	0	0 (2)	62*	62 (64)
All nodes		13	2 (4)	63	78 (80)
Per patient					
	++	6	2	0	8
OSNA	+	0	0	1	1
	–	0	0 (2)	35*	35 (37)
All patients		6	2 (4)	36	44 (46)

Values in parentheses indicate case numbers before discordant case investigation, *2 cases with isolated tumour cells.

Table III. *Statistical data of the clinical study phase.*

Statistical factor (%)	Per node		Per patient	
	Before DCI	After DCI	Before DCI	After DCI
Specificity	98.4	98.4	97.2	97.2
Sensitivity	88.2	100	80.0	100
Concordance	96.3	98.7	93.5	97.7
NPV	96.9	100	94.6	100
PPV	93.8	93.8	88.9	88.9

NPV, Negative predictive value; PPV, positive predictive value.

Table IV. *Routine OSNA use versus 1 level histology.*

		Histology (1 level)				
		Macro-metastases	Micro-metastases	Negative	All	Non-SLN positivity rate
OSNA	++	9	1	3Δ	13	54%
	+	8	7	14*	29	10.3%
	–	0	0	155*	155	
All patients		17	8	172	197	

Positivity rate=21.3%. Δ one case=pN0(i+) (OSNA ++/histology negative) and NSLN positive; one case=false positive due to unvalid controls during the run (OSNA ++/histology negative); one case=OSNA ++/histology negative and NSLN positive. *one case=pN0(i+).

Table V. *Evaluation of OSNA for the detection of lymph node metastases in breast cancer patients: results per lymph node and after DCI unless indicated differently**.

Study	SLNs	Concordance (%)	Specificity (%)	Sensitivity (%)
Tsujimoto *et al*. (13)*	325	98.2	100	95.6
Visser *et al*. (14)	346	96.8 (94.8)	97.1 (94.7)	95.3 (95.3)
Schem *et al*. (15)	343	95.5 (91.8)	95.6 (90.8)	100 (98.1)
Tamaki *et al*. (16)*	450	92.9	94.1	87.5
This study	80	98.7 (96.3)	98.4 (98.4)	100 (88.2)

*Only results before DCI available; QRT-PCR was not performed in these studies. Values in parentheses indicate case numbers before DCI.

domain containing ETS transcription factor, *SPDEF*, forkhead box A1, *FOXA1*) as well as beta-actin for RNA control as described elsewhere (14, 17-18). RNA was extracted from 200 μl of the homogenate. The cut-off levels for each marker were determined according to the QRT-PCR results of a series of histologically positive and negative lymph nodes from breast cancer patients.

Results

During the study phase, 80 SLNs from 46 patients were analysed (mean=1.8 nodes per patient). In 62 SLNs and 35 patients, both OSNA and histology yielded negative results (Table II). OSNA detected all the macrometastases and 2/4 micrometastases. Two patients with micrometastases detected by permanent histology were found to be negative by OSNA (Table II). In one case, the micrometastases was confined to 2 out of the 5 levels of histology, in the other case the micrometastases were present in slice b whereas slice d was metastasis-free. In the one OSNA positive/histology negative case, a (+) result with 380 *CK19* mRNA copies/μl was obtained which was very close to the cut-off level (250 *CK19* mRNA copies/μl) of the molecular assay, indicating very small tumour deposits. The statistical data obtained before DCI are presented in Table III.

Further molecular analysis of the two histologically positive/OSNA negative cases also showed negative results in QRT-PCR with three markers so it was concluded that the discordance was caused by TAB. The OSNA positive/histologically negative sample with a CK19 low copy number gave a negative result in DCI. If the two cases (nodes as well as patients) with TAB were excluded from the cohort, specificity was (62/63) 98.4%, sensitivity (15/15) 100%, and concordance (77/78) 98.7% on a per node basis and (35/36) 97.2%, (8/8) 100%, and (43/44) 97.7%, respectively, on a per patient basis (Table III). The PPV of OSNA per patient was 88.9% and the NPV 100% after DCI.

During routine use, 25 metastases indicated by OSNA were also detected by permanent histological examination of the central slice (Table IV). Seventeen patients were found to be

positive exclusively by OSNA: 3 (++) cases had NSLN involvement and 14 (+) cases indicated a micrometastases tumour burden, which could explain the negative result on the 1 mm central slice. For 41/42 patients, presenting a positivity rate of 21.3%, axillary clearance was performed during the same surgical session as the SLN biopsy. One patient woke up before the OSNA analysis was finished so ALND had to be performed in a different surgical session. The positivity rate of NSLNs in the patients with a (++) OSNA result was 54% and in patients with a (+) result was 10.3%.

In our hands, the median time of analysis was 37 minutes for two SLNs, from the reception of the SLN in the pathology laboratory to the result.

Discussion

The performance of OSNA in comparison to serial sectioning with H&E staining and CK IHC performed at the St. Etienne University Hospital showed 98.7% concordance, 98.4% specificity and 100% sensitivity per node after DCI and supported the excellent data obtained in other clinical studies (Table V) (14, 17-19). In the clinical study, two out of the three discordant results were caused by TAB due to the use of alternating slices for each method of analysis (23).

The RD-100 instrument software classifies data into qualitative categories, further described by quantification of *CK19* mRNA copy number, enabling an objective and standardised interpretation by any technician or clinician after brief familiarisation with the technique. Since no RNA purification is required and pipetting plus mixing of reagents or samples for *CK19* mRNA amplification is carried out by an automatic pipetting device, the number of manual steps involved in the OSNA procedure is quite small, assuring reproducible tissue and assay management. OSNA was readily performed after a short training phase and was brought into routine use after the end of the clinical study. In our institute, the complete analysis time from the arrival of the SLN to the result took 37 minutes for two SLNs.

Due to the fact that the whole SLN except the central 1 mm slice was analysed by OSNA, the proportion of uninvestigated material and associated risk of sampling errors was reduced to a minimum. However, since a larger proportion of each SLN was submitted to OSNA, discordant cases were still expected. Interestingly, two out of the three OSNA (++)/histologically negative cases revealed NSLN involvement. The positivity rate of 21% for OSNA was a little lower in comparison to the histological work-up (positivity rate of 25%) used before the molecular diagnostic tool was introduced: cutting the SLN into 2 mm slices with 3 levels (150 μm skip ribbon in between) of H&E and IHC performed for each slice. The rate of NSLN involvement was 54% for patients with a macrometastasis in the SLN detected by OSNA which was comparable to data reported in the literature (24). The rate of NSLN involvement was 10.3% for the patients with a micrometastasis in the SLN, which was similar to other reports of 9.3% (24) to 13.4% (25). By applying OSNA as an intra-operative routine, 16 patients avoided a recall for second ALND, thereby improving the patient benefits and at the same time saving costs and shortening the pathologist's time spent on post-operative SLN analysis.

In conclusion, OSNA is a rapid, simple, and accurate tool for intra-operative assessment of SLN.

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