Using one-step nucleic acid amplification (OSNA) for intraoperative detection of lymph node metastasis in breast cancer patients avoids second surgery and accelerates initiation of adjuvant therapy

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Background: Sentinel lymph node (SLN) analysis is conventionally analyzed using immunohistochemistry and in the case of SLN involvement, justifies a second surgery for axillary lymph node (ALN) resection, thus delaying the initiation of adjuvant therapies.

Patients and methods: Three hundred and eighty-one patients with early stage breast cancer (BC) were considered in this retrospective study. SLNs were detected using combined radioisotope and dye detection. SLN involvement was analyzed using routine intraoperative One-Step Nucleic Acid Amplification (OSNA) assay, in 100 patients and compared with the conventional histopathology carried out previously in 281 patients.

Results: Considering positive SLNs as ‘++’ (CK19 mRNA copy number>5000), ‘+’ (250 < CK19 mRNA copy number <5000) and positive by inhibition in the OSNA group and macro-, micrometastases and isolated tumor cells in the histopathology group, no difference in SLN involvement rate was found between the two groups with 29.0% and 29.9% of positive SLNs, respectively. Using OSNA intraoperatively, the mean time to process the SLN was 42 min allowing immediate ALN resection, reduced significantly (P<0.01) the re-intervention rate (9% versus 39%) and significantly (P<0.01) accelerated the initiation of adjuvant therapy (6.2 versus 8.4 weeks).

Conclusions: Using OSNA for intraoperative SLN analysis avoids second surgery for ALN resection in most patients and accelerates initiation of adjuvant therapy.

Key words: breast cancer, CK19 mRNA, intraoperative assay, OSNA, PCR assay, sentinel lymph node

introduction

Sentinel lymph node (SLN) analysis has become a standard of care in early-stage breast cancer (BC) [1, 2] and avoids immediate axillary lymph node (ALN) resection in disease-free SLN patients, therefore reducing arm morbidity and improving the quality of life [3]. The most conventional way of analysis of SLNs is post-operative histopathology. Other techniques, such as imprint and frozen section analysis, can be used intraoperatively, thus enabling reduction of second surgery. Because of the poor sensitivity of touch imprints [4] and the tissue loss induced by frozen section analysis, sometimes impairing postoperative paraffin-embedded tissue analysis, the gold-standard remains multistep analysis of hematoxylin–eosin stained sections, most frequently combined with immunohistochemistry for cytokeratin expression detection. During the last decade, PCR-based techniques have been proposed and the one-step nucleic acid amplification (OSNA) assay is now increasingly used. The OSNA assay is based on reverse transcription isothermal loop-mediated DNA amplification for the detection of cytokeratin 19 (CK19) messenger RNA (mRNA). CK19 has been reported to be expressed in most BC.

Many studies have compared the OSNA assay with frozen sections and immunohistochemistry and yielded comparable results (for review, see 5) and validated OSNA for routine intraoperative SLN analysis. When observed, the rare discrepancies were mostly attributed to micrometastases strictly located in SLN volume analyzed by only one of the two techniques. Pooled analysis [5] of recent studies indicated that OSNA is as accurate as pathology with 96.3% concordance rate...
and is useful for making decisions to avoid ALN resection in OSNA-negative patients (97.4% negative predictive value).

The OSNA assay is CE approved and used routinely in many hospitals. The procedure used in this study has previously been validated through the participation of our institute in the multicentric French validation study [6]. Indeed, being carried out intraoperatively, the main advantage of the OSNA assay is to avoid second surgery in case of positive SLN involvement. Besides the positive impact on the quality of life of patients [7, 8], the OSNA assay provides an additional economical interest, consisting of sparing surgical room and hospitalization extra-costs. Avoiding second surgery also allows quicker initiation of adjuvant therapy in SLN-positive patients. This is a major issue since the initiation of adjuvant therapy before 12 weeks after surgery has been reported to increase recurrence-free and overall survival in early-stage BC [9] and overall survival in metastatic BC patients [10]. Another advantage of OSNA is to be a quantitative method, providing the thresholding of SLN involvement to address whether all positive SLNs have a high risk of metastatic spreading that justify ALN resection and adjuvant treatment with all the associated morbidities. As recently reported by the American Society of Breast Surgeons [11], molecular biology assays based upon the analysis of the whole SLN could be predictive of non-SLN metastases and thresholding CK19 mRNA copy number could provide a rational way for omitting ALN resection in patients with one or two positive SLNs treated with tangential radiotherapy. The present study was designed to quantify the impact of routine intraoperative SLN analysis using the OSNA assay on the second surgery rate and delay before initiation of adjuvant chemotherapy when compared retrospectively with the conventional analysis of SLN using immunohistochemistry in a historical cohort of patients with early-stage BC.

### patients and methods

#### patients

From August 2005 to June 2011, 381 patients with pT1-2 BC underwent SLN biopsy. This population was subgrouped in 100 patients in which SLN biopsy was analyzed using an intraoperative OSNA assay, and 281 patients with conventional histopathology analysis. When SLN was positive with OSNA, ALN resection was carried out during the same surgery time. These two groups were well balanced for most tumor and patient characteristics (supplementary Table S1, available at *Annals of Oncology* online).

#### SLN biopsy procedure

The SLNs were localized using an isotopic method alone or mostly combined with the dye method. The isotopic method consisted of 99mTc-labeled rhenium sulfur (Amersham, France) subareolar injection the day before surgery followed by lymphoscintigraphy 2–4 h later. The dye procedure consisted of subareolar injection of 1.5–2 ml Patent Blue dye (Guerbet, Aulnay sous Bois, France) at surgery.

SLNs were identified using a handheld gamma-probe (Europrobe 2, Euromedical Instruments, Le Chesnay, France) with, when available, the assistance of vessel and lymph node staining. Failure of SLN identification was encountered in 2.1% of the cases.

### histopathology of SLN

Each SLN was measured, cut longitudinally into 2 mm sections and entirely embedded in paraffin. Every section was then cut into three ribbons with a 150-µm skip space. From each ribbon, two sections were prepared, one for hematoxylin and eosin staining and one for IHC with pan-CK antibody AE1/AE3 (Dako), processed on Benchmark automat (Ventana). Histopathology categories were defined according to the sixth edition of the TNM [12].

#### OSNA assay

The OSNA assay was processed as previously reported [6], using the OSNA BC System (Sysmex, Kobe, Japan). Briefly, after removing extranodal and fatty tissue, the SLNs were homogenized in Lyncorhag lysis buffer then processed according to the manufacturer’s instructions. SLNs exceeding the specified maximum weight of 600 mg were cut into two or more pieces and processed separately. CK19 mRNA expression was quantified using an RD-100i analyzer. The maximal loading capacity was four SLNs per run. Each run lasted approximately 20 min.

To avoid any false-negative results due to the lack of CK19 expression in the tumor tissue, all surgical resection pieces were analyzed for CK19 expression using IHC (RCK108 antibody, Dako). Only two negative cases were found within OSNA group patients (data not shown).

#### statistics

A Khi2 test was used to compare qualitatively the clinical data and histopathological characteristics of the two subgroups. Quantitative comparisons between the two groups were carried out using Student’s *t* test. Breslow, Day and Tarone tests was used to test for homogenicity of metastasis detection. In all cases *P* < 0.05 was used as significance level.

### results

#### duration of OSNA intraoperative procedure

After removal, SLNs were immediately shipped pneumatically from the operating ward to the biopathology department and processed less than 10 min after excision. Within the laboratory, the mean duration of the OSNA procedure, including reception of the SLNs, macrodissection and fatty tissue removal, validation, Q-PCR and data processing, results validation and their transmission to the surgeon, was 43 min (range 30–94) depending on the number of SLN.

#### comparative analysis between OSNA and histopathology groups

A mean number of 2.4 SLNs (range 1–8) was removed per patient in OSNA group and 2.5 (range 1–8) in histopathology group (*P* = 0.41). No difference in SLN involvement rate (Table 1) was observed between OSNA (29.0%) and histopathology group whether the cases with isolated tumor cells, were considered as positive (29.9%) or not (24.2%). No difference was observed between OSNA + and +I (15.0%), and macrometastases (11.7%) rates as well as OSNA+ (14.0%) and micrometastases (12.5%) rates.

#### patients with SLN involvement

The patient population was subgrouped according to SLN involvement status in both OSNA and histopathology groups (supplementary Table S2, available at *Annals of Oncology*).
online. The two groups were comparable for age, tumor size and histological type, SBR grade, hormone receptors and HER2 status. Although not significant, the rate of SLN involvement seemed to be higher in the OSNA group for patients with pT1a tumors (50% versus 15% in the histopathology group).

**Second surgery**

The second surgery rate (Table 2) was calculated by considering surgery for ALN dissection, recovery of margins, or both. A second surgery was performed in 9 patients (9%) in the OSNA group and in 109 patients (38.8%) in the histopathology group showing that using OSNA reduces dramatically the second surgery rate ($P = 0.01$). Three patients had a second surgery for ALN resection in OSNA group as compared with 43 (15.3%) in the histopathology group. The higher rate of second surgery for margins alone observed in the histopathology group was most probably related to modification of the surgery procedures during the time of the study, as the margin limit was changed from 5 mm to 2 mm, according to the 2009 French National Cancer Institute guidelines for infiltrating breast carcinoma.

**Non-sentinel lymph nodes (NSLN) metastases**

Considering the whole population of patients, in which the SLN was positive according to both OSNA and histopathological criteria, ALN resection was carried out in 114 patients (29 in OSNA group and 84 in histopathology group). No difference was observed between OSNA group and histopathology group comparing OSNA ++ and +I versus macrometastases and OSNA + versus micrometastases. As a whole, NSLN invasion was observed (Table 3) in 19 cases (16.8%). The rate of NSLN metastases was higher in the macrometastasis or OSNA ++/+I subgroup (23.0%) than in the micrometastasis or OSNA+ subgroup (15.6%) and in the isolated tumor cells subgroup (8.3%) illustrating the relation underlying the degree of SLN involvement and the presence of NSLN metastases.

**Delay before adjuvant therapy for patients with positive SLN**

The delay before adjuvant therapy was calculated as the time between initial surgery and the onset of chemotherapy, radiotherapy, hormonal therapy or the second surgery if it was carried out after chemotherapy. The mean delay was significantly ($P = 0.004$) lower in the OSNA group (6.2 weeks, CI95 7.4–9.4) than in the histopathology group (8.4 weeks, CI95 5.5–6.9), enabling an earlier (2.2 weeks, CI95 0.5–3.9) onset of adjuvant therapy.

**Discussion**

In most hospitals, postoperative histopathology is considered as the gold standard for SLN analysis. However, to reduce the false-negative rate to ~5% and warrant the detection of small-volume metastases including isolated tumor cells requires the exhaustive SLN examination and the use of immunohistochemistry for cytokeratins. Using such a procedure is laborious and time-consuming, requires second surgery when SLNs are involved and consequently delays the initiation of adjuvant treatment. Intraoperative alternatives exist as imprints or frozen sections analysis but suffer from lack of sensitivity or excessive laboriousness if used for thorough SLN analysis. Therefore, molecular biology methods enabling highly sensitive intraoperative diagnosis of SLNs are attractive, analyzing the whole SLN within delays that authorize immediate ALN resection in case of positivity. The OSNA assay fulfills these criteria. In all validation studies (for review, see 5), intraoperative OSNA proved to be highly sensitive, specific, reproducible and suitable for SLN diagnosis standardization with an acceptable turnaround time. In these studies, attention was focused on the fact that the larger portion of the SLN that is excluded from the OSNA analysis increases the risk that micrometastases might have missed, thus generating discordant results.

**Table 1. SLN involvement using OSNA or conventional histopathology**

<table>
<thead>
<tr>
<th>OSNAa</th>
<th>n</th>
<th>%</th>
<th>CI 95%</th>
<th>Histopathologyb</th>
<th>n</th>
<th>%</th>
<th>CI 95%</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>29</td>
<td>29.0%</td>
<td>20.1% to 37.9%</td>
<td>Positive</td>
<td>84</td>
<td>29.9%</td>
<td>24.5% to 35.0%</td>
<td>0.35</td>
</tr>
<tr>
<td>(+)/(+I)</td>
<td>15</td>
<td>15.0%</td>
<td>8.0% to 22.0%</td>
<td>Macro-metastases</td>
<td>33</td>
<td>11.7%</td>
<td>8.0% to 15.6%</td>
<td></td>
</tr>
<tr>
<td>(+)</td>
<td>14</td>
<td>14.0%</td>
<td>7.2% to 20.1%</td>
<td>Micro-metastases</td>
<td>35</td>
<td>12.5%</td>
<td>8.6% to 16.3%</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>71</td>
<td>71.0%</td>
<td>62.1% to 79.9%</td>
<td>Isolated cells</td>
<td>16</td>
<td>5.7%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>71</td>
<td>70.1%</td>
<td>64.7% to 75.5%</td>
<td>Negative</td>
<td>197</td>
<td>70.1%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The cut-off levels of calculated CK19 mRNA copy numbers/µL were applied according to the manufacturer’s instructions as negative (<2.5 × 10^2 copies/µL), + positive (>2.5 × 10^2 and <5.0 × 10^3 copies/µL), ++ positive (>5.0 × 10^3 copies/µL) or positive +I (inhibited in the regular sample and >2.5 × 10^2 copies/µL in the diluted sample).

**Table 2. Second surgery**

<table>
<thead>
<tr>
<th>Second surgery</th>
<th>OSNA</th>
<th>Histopathology</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>Margins</td>
<td>5</td>
<td>5.0%</td>
<td>36</td>
</tr>
<tr>
<td>ALN resection</td>
<td>3</td>
<td>3.0%</td>
<td>43</td>
</tr>
<tr>
<td>Both</td>
<td>1</td>
<td>1.0%</td>
<td>30</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>9.0%</td>
<td>109</td>
</tr>
</tbody>
</table>
Further, beyond the fact that intraoperative analysis of SLN involvement and the presence of metastases in the resected whole SLN. A semi-quantitative assessment of the tumor volume in the isolated tumor cells were considered as positive results, giving a very strong agreement. OSNA assay can provide reliable results for micrometastases which SLNs were more tightly fractionated [13] or near-entirely isolated tumor cells, since an excellent agreement is seen to come out as a weaker test for the identification of micrometastases [16]. When compared with intraoperative frozen sections analysis [17], the OSNA assay was reported to detect more SLN metastases, particularly micrometastases, especially in elderly patients or in patients with less progressive primary tumors. Recently [18], this was also reported in breast ductal carcinoma in situ.

Comparing the results achieved with routine use of the OSNA assay and a historical histopathology series, the present study appears to be close to Godey et al. [19] and cannot yield specificity and sensitivity results but only rates of positive and negative cases per patient. As a whole, we show no difference between OSNA and histopathology, and report positivity rates, which are fully consistent with those reported in OSNA validation studies [5] as well as in a recent study carried out in selected BC with >80% CK19 expressing cells in the primary tumor [20]. In addition, our study further confirms that the OSNA assay can provide reliable results for micrometastases and probably isolated tumor cells, since an excellent agreement was found between OSNA and histopathology results when isolated tumor cells were considered as positive results, giving a semi-quantitative assessment of the tumor volume in the whole SLN.

Although the thresholds used in the OSNA assay differ by nature from the criteria used in histopathology, our results confirm the relationship between the degree of SLN involvement and the presence of metastases in the resected NSLN as reported in the meta-analysis of Van la Parra et al. [21]. Further, beyond the fact that intraoperative analysis of SLN may avoid second surgery, we clearly demonstrate that using intraoperative OSNA ensured that the patients with positive SLNs can receive adjuvant treatment within 7 weeks, with 95% chance. Economically, based upon a survey of our institute, OSNA is slightly less expensive (1.9%) than histopathology (unpublished data). One disadvantage of OSNA remains the complete destruction of the SLN, thus prohibiting any additional microscopic assessment of the SLN and diagnosis of diseases that may rarely affect the SLN.

In conclusion, the OSNA assay of SLN enables quantitative categorization of the metastasis, making the system attractive for routine use when compared with other intraoperative techniques. OSNA is also being investigated in gastric [22], colon [23] and head and neck cancer [24, 25] and for post-operative staging of resected axillary NSLN [26, 27] in BC. Follow-up of patients with regard to quantitative results achieved with OSNA assay could contribute to innovative BC staging for personalized decision making for adjuvant treatment.

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**disclosure**
The authors have declared no conflicts of interest.

**references**

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**Table 3. Non-sentinel lymph node (SLN) metastasis in resected axillary lymph nodes (ALNs) in patients with positive SLN**

<table>
<thead>
<tr>
<th>Non SLN metastasis</th>
<th>Macrometastasis/OSNA ++ and +I</th>
<th>Micrometastasis/OSNA +</th>
<th>Isolated cells*</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>Negative</td>
<td>37</td>
<td>77.0</td>
<td>38</td>
<td>84.4</td>
</tr>
<tr>
<td>Positive</td>
<td>11</td>
<td>23.0</td>
<td>7</td>
<td>15.6</td>
</tr>
<tr>
<td>Not carried out</td>
<td>0</td>
<td></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td>48</td>
<td></td>
<td>49</td>
<td></td>
</tr>
</tbody>
</table>

*Isolated tumor cells criteria was only applicable to the histopathology group.*


