Intraoperative diagnosis of sentinel lymph node metastases in breast cancer treatment with one-step nucleic acid amplification assay (OSNA)

Pawel Szychta1, Boguslaw Westfal1, Rafal Maciejczyk1, Beata Smolarz2, Hanna Romanowicz2, Tomasz Krawczyk2, Marek Zadrozny1

1Department of Oncological Surgery and Breast Diseases, Institute of Polish Mother’s Memorial Hospital, Lodz, Poland
2Laboratory of Molecular Genetics, Department of Pathology, Institute of Polish Mother’s Memorial Hospital, Lodz, Poland

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Abstract

Introduction: The aim of the study was to evaluate the clinical usefulness of a one-step nucleic acid amplification assay (OSNA) for intraoperative detection of metastases to sentinel lymph nodes (SLNs) in comparison to examination of frozen sections, and to summarize the results of previous studies.

Material and methods: We enrolled 98 patients aged 58.13 ±10.74 years treated surgically for breast cancer, and 99 biopsies of SLNs were followed by analysis of 105 SLNs. The central 1 mm slice of SLN was used for examination of frozen sections, whereas 2 outer slices of SLNs were analyzed intraoperatively with OSNA. Detection of isolated tumor cells (ITC), micrometastases or macrometastases with OSNA extended surgery to axillary lymph node dissection. Congruency of results was assessed between OSNA and examination of frozen sections.

Results: One-step nucleic acid amplification assay detected metastases in 29/105 SLNs in surgery of 27/99 breasts, including ITC in 3/29 SLNs, micrometastases in 12/29 and macrometastases in 14/29. One-step nucleic acid amplification assay detected significantly more metastases to SLNs than examination of frozen sections (p < 0.0001). All 8 inconsistent results were positive in OSNA and negative in examination of frozen sections; ITC were identified in 2/8 SLNs and micrometastases in 6/8 SLNs. Sensitivity for OSNA was calculated as 100%, specificity as 90.47%, and $\kappa$ was 79.16%.

Conclusions: One-step nucleic acid amplification assay analysis allows rapid and quantitative detection of mRNA CK19 with high specificity and a low rate of false positives. One-step nucleic acid amplification assay is a reliable tool for intraoperative diagnosis of whole SLNs during surgery of breast cancer. One-step nucleic acid amplification assay minimizes the need for secondary surgery and avoids delays in the adjuvant treatment.

Key words: breast cancer, sentinel lymph node, intraoperative molecular study, cytokeratin 19.

Introduction

Intraoperative evaluation of sentinel lymph nodes (SLNs) in the surgical treatment of breast cancer is based on microscopic examination
of frozen sections, which unfortunately does not allow for accurate diagnosis in all cases [1].

Recently described intraoperative molecular methods for the detection of metastatic breast cancer to SLN detect mRNA expression of the epithelial marker cytokeratin 19 (CK19) [2–6]. CK19 is normally absent in the lymph node and its high level of expression is seen in the majority of breast cancer cells. The number of copies of mRNA for CK19 can be assessed with the one-step nucleic acid amplification test (OSNA). Sensitivity and specificity of the OSNA test for the detection of metastases has been shown in a few studies using different research protocols [7–10]. The results of previous reports should be summarized in order to propose standards for treatment of breast cancer together with validation of the intraoperative SLN evaluation using OSNA in the clinical setting.

The aim of this study was to evaluate the clinical usefulness of OSNA for the intraoperative detection of metastasis in SLN in comparison to the examination of frozen sections with H + E staining, as well as to summarize the results of previous research in this area.

**Material and methods**

A prospective study was conducted from May 2011 to July 2012 after the approval of the Ethics Committee of the Institute of Polish Mother’s Memorial Hospital, Lodz, Poland. Inclusion criteria were: preoperatively diagnosed monofocal breast cancer at stage less than T3, no neoadjuvant systemic chemotherapy or hormone therapy, no previous surgery to the affected breast in the past and no clinically detected metastases (Tis-T2N0M0, according to the 6th edition of the TNM classification) [11]. One patient with pT3 took part in the study because the tumor size was underestimated before surgery.

Preoperatively patients were subjected to examination of the axilla with ultrasound (US), which was part of staging. On the day of surgery we administered subcutaneously 2 ml of blue dye (2.5% Patentblau V, Guerbet, Aulnay-sous-Bois, France) above the tumor [12]. After a mastectomy or breast conserving surgery SLNs were identified and removed using a well-known surgical technique. Up to 4 SLN were identified and removed per patient (1.12 sentinel nodes on average).

The central 1 mm slice of SLN was removed with a Sysmex cutting device and was used for examination of frozen sections with H + E staining (Figure 1). The two outer slices of the node were analyzed intraoperatively with a OSNA. The method of analysis of CK19 mRNA expression using OSNA has been previously described in detail [10]. After purification from the surrounding tissues, lymph nodes are homogenized in the mRNA stabilizing solution Lynorhag (Sysmex). The resulting homogenate is subjected to isothermal amplification (65°C) of cytokeratin 19 (CK19) for 16 min with a Lynoamp kit (Sysmex) through a reverse transcriptase amplification test (RT-LAMP) in the amplification detector RD-100i (Sysmex) [13]. The isothermal reaction prevents amplification of genomic DNA, which would give false positive results. Sensitivity, specificity, and speed of the test are high when using six primers [14]. Samples are diluted with Lynorhag solution (1 : 10) to inhibit the activity of substances inhibiting the amplification (such as adipose tissue) [6]. The analysis can be performed on up to four parallel lymph nodes. Total time from preparing the specimen to obtaining results is 30 min for one lymph node and 40 min for four lymph nodes [15]. The qualitative result of CK19 (negative or positive) and quantitative result (number of copies of CK19 mRNA/1 µl lysate) determined the status of the lymph nodes: number of copies < 250 = no metastases (qualitative result: negative "−", quantitative result < 250) or isolated tumor cells (ITC) (positive "+", < 250), number of copies 250–5000 = micrometastases (positive "++", 250–5000), and number of copies > 5000 = macrometastases (positive "+++", > 5000).

Intraoperative analysis of the SLN using OSNA determined the further surgical treatment. Isolated tumor cells (ITC), micro- and macrometastases described the SLN as positive (SLN+). In the case of SLN+, the operation was extended to further axillary lymph node dissection (ALND) [12]. The breast specimen with a tumor was sent for postoperative histological examination with assessment of the activity of estrogen and progesterone receptors and HER2.

The electronic database included: the patient’s personal data, histological diagnosis of the tumor, staging according to the sixth edition of the TNM classification, grading, the number of isolated SLNs, diagnosis from examination of frozen sections, diagnosis using OSNA, number of mRNA CK19 copies/µl detected by OSNA, number of lymph nodes removed in ALND, activity of estrogen and progesterone receptors, HER2 expression and presence of

![Figure 1. A 1-mm-thick slice was cut out from the longitudinal central part of the sentinel lymph node for staining with hematoxylin and eosin (H + E), and the remaining parts were examined by using the one-step nucleic acid amplification (OSNA) assay](image-url)
metastases in non-sentinel lymph nodes. Congruency of the results was assessed between OSNA and microscopic evaluation of frozen sections. A thorough analysis of cases was conducted where axillary lymphadenectomy was performed.

Compliance or inconsistency of the SLN metastases between results of the OSNA test and the microscopic examination of frozen sections was calculated using Cohen’s “κ” index. Sensitivity, specificity, positive and negative predictive values were calculated for the OSNA method with the McNemar test. Analysis of two dichotomous variables was performed with the χ² test.

**Statistical analysis**

Statistical significance between a continuous variable and a dichotomous variable was assessed using the logistic regression test. Values of p < 0.05 were considered statistically significant.

**Results**

In 111 patients, 112 sentinel lymph node biopsies (SLNBs) were performed and were subjected to histological and molecular evaluation of 121 SLNs. However, in 4 patients (6 SLNs) we could not obtain results of histological examination of frozen sections, and in 9 cases (10 SLNs) all SLNs were evaluated using only OSNA. Therefore, the study ultimately included 98 patients aged 58.13 ±10.74 years (29 to 87 years). Patients had 1.07 SLNs resected on average (1–2 SLNs), and one woman who had bilateral breast cancer underwent bilateral SLNB. Therefore, 105 SLNs were assessed in the study from 99 SLNBs. For all specimens, OSNA assay and microscopic examination of frozen sections were performed successfully.

Radical modified mastectomy was undertaken for 25/99 (25.25%) breasts, quadrantectomy in 42/99 (42.42%) cases and a wide local excision in 32/99 (32.32%). Histopathological examination detected the following types of breast cancer: ductal carcinoma in situ in 6/99 (6.06%), invasive ductal carcinoma in 74/99 (74.74%), lobular carcinoma in situ in 2/99 (2.02 %), invasive lobular carcinoma in 5/99 (5.05%), mucinous carcinoma in 4/99 (4.04%) and other in 8/99 (8.08%). According to the classification of cancer staging, Tis was observed in 8/99 (8.08%), T1 in 70/99 (70.70%), T2 in 20/99 (20.20%) and T3 in 1/99 (1.01%). Based on the Bloom-Richardson grading scale, grade I was observed in 26/99 (26.26%), II in 45/99 (45.45%), III in 7/99 (7.07%), it was unknown in 13/99 (13.13%) and it was not evaluated in 8/99 (8.08%). Detailed information on the activity of estrogen receptor and progesterone receptor, HER2 expression, vascular invasion and lymphatic invasion is shown in Table I.

Microscopic examination of frozen sections with H + E staining detected metastases to SLN in 21/105 (20.00%) specimens. In the OSNA assay a positive result (metastasis) was obtained in 29/105 (27.27%) SLNs excised during surgery of 27/99 (27.27%) breasts, including isolated tumor cells (ITC) in 3/29 (10.34%) SLNs, micrometastases (“+”) in 12/29 (41.37%) and macrometastases (“++”) in 14/29 (48.27%) (Table II). Thus, the OSNA test detected significantly more breast cancer metastases to the SLNs than histological examination of frozen sections (χ² = 68.79, p < 0.0001), despite the fact that the central slice of each SLN was examined by microscopic examination. Of the 8 inconsistent results of SLN status, they were all positive in OSNA and negative in the histological examination of frozen sections (O+/H–), and in all cases the primary tumor was invasive ductal carcinoma. Among 8 SLNs O+/H–, isolated tumor cells (ITC) were identified in 2/8 (25%) sentinel nodes, and micrometastases in 6/8 (75%) cases. As a result, the sensitivity was calculated for OSNA as 100%, specificity as 90.47%, and κ was 79.16% (Table III).

The ALND was performed during surgery of 29/99 (29.2%) breasts as a result of the positive SLN result in OSNA (Table IV). Metastases to non-SLNs were found in 1/3 (33.3%) cases of SLNs assessed as OSNA-“+” ITC, in 4/12 (33.3%) cases detected as OSNA-“+” micrometastasis and in 5/14 (35.7%) who had OSNA-“++” results. Thus, the risk of non-SLN metastases was insignificantly higher for OSNA-“++” results compared to OSNA-“+” overall (p > 0.05).

| Parameter | Positive (> 10%) | Negative (< 10%) | Unknown |
|-----------|----------------|----------------|---------|
|           | n   | %   | n   | %   | n   | %   |
| Estrogen receptor | 64/81 | 79.01 | 17/81 | 20.98 | 18/99 | 18.18 |
| Progesterone receptor | 59/81 | 72.83 | 22/81 | 27.16 | 18/99 | 18.18 |
| HER2 | 26/76 | 34.21 | 50/76 | 65.78 | 23/99 | 23.23 |
| Vascular invasion | 3/91 | 3.29 | 88/91 | 96.70 | 8/99 | 8.08 |
| Lymphatic invasion | 4/91 | 4.39 | 87/91 | 95.60 | 8/99 | 8.08 |
Discussion

Reliable intraoperative SLN assessment is invaluable for optimal surgical treatment of breast cancer. In the case of breast cancer metastasis to the SLN, ALND is performed simultaneously with the primary breast tumor surgery so that the patient avoids further surgery and adjuvant therapy is initiated promptly [12]. In addition, simultaneous ALND is advantageous as there is a high possibility to remove all axillary lymph nodes from the previously unchanged tissues, resulting in high reliability of the method. In contrast, delayed ALND is associated with lymph node dissection in the scar tissue formed after previous SLNB. Intraoperative analysis of the SLN is economically advantageous, and the implicit savings result from the reduced number of hospitalizations and avoided secondary surgery, which compensates for the cost of the intraoperative OSNA assay [16].

In our study, microscopic examination of SLNs from frozen sections was compared with the OSNA test to detect a more reliable method supporting the intraoperative decision of simultaneous ALND. Histological examination of frozen sections assesses SLN only in the selected two-dimensional sections with the risk of false-negative results, particularly in the case of micrometastases [3, 17]. There are also difficulties in the diagnosis of metastases to SLNs in invasive lobular carcinoma [18]. Accordingly, the sensitivity of the intraoperative histological examination has been designated for macrometastases at a high level from 84% to 94%, while for micrometastases there is a wide range from 17% to 92% [1]. In about 20% of cases with a negative SLN result of the intraoperative histological examination, a positive result of postoperative full histological examination resulted in deferred ALND [14].

Therefore the advantage of SLNB is accurate staging classification and detection of patients requiring ALND. If patients with SLN metastases have missed early ALND, they are prone to breast cancer recurrence and/or distant metastases because of the risk of non-SLN metastases. In our study, analysis of SLN showed ITC or micrometastases in 14% of cases and macrometastases in 13%, and all of these patients required ALND with adjuvant systemic therapy. Histological examination of axillary nodes showed metastatic non-SLNs in 33% of cases with an OSNA-"++" result (ITC and micrometastases) and 35% of patients with OSNA-"+++". Previous reports described non-SLNs metastases in the range from 13% to 22% of patients with SLN micrometastases and from 45% to 79% of patients with SLN macrometastases [19].

Conversely, SLNB also aims to select patients who do not require ALND. As previously reported, approximately 60% of patients with positive SLNs

Table II. Relationship between parameters describing breast tumor and metastases to SLN found with OSNA

| Parameter | Metastases (overall) | ITC | Micrometastases | Macrometastases |
|-----------|---------------------|-----|----------------|-----------------|
| Frozen section result | 70.922 | – | 0.32 | 0.17-24.45 | 0.0001 |
| Breast tumor stage | 2.70 | 0.86-4.50 | NS | 0.32 | 0.21-5.36 |
| Breast tumor grade | 2.86 | 0.88-4.63 | NS | 0.50 | 0.28-1.74 |
| Invasive ductal carcinoma | 5.55 | 1.47-26.02 | 0.001 | 0.01 | 0.16-3.30 |
| ER | 0.04 | 0.39-1.20 | NS | 0.22 | 0.06-1.36 |
| PR | 0.06 | 0.35-2.24 | NS | 0.16 | 0.06-1.13 |
| HER2 receptor | 5.39 | 1.08-28.00 | 0.002 | 0.01 | 0.01-3.86 |
| Lymphatic invasion | 4.03 | 0.83-89.43 | 0.0445 | 0.24 | – |
| Vascular invasion | 1.88 | 0.46-8.96 | 0.18 | – | – |

OR – odds ratio, ER – estrogen receptor, PR – progesterone receptor.
Table III. Contingency table and concordance between OSNA assay results and \( \chi^2 = 68.79, p < 0.0001 \)

| Result of SLN assessment | OSNA |  |
|--------------------------|------|-----|
|                          | Positive | Negative | Total |
|                          | \( n \) | %   | \( n \) | %   | \( n \) | %   |
| Positive                 | 21/105  | 20.00| 0/105  | 0.00| 21/105  | 20.00|
| Negative                 | 8/105   | 7.61 | 76/105 | 72.38| 84/105  | 80.00|
| Total                    | 29/105  | 27.61| 76/105 | 72.38|        |      |

Table IV. Risk of non-sentinel lymph node metastasis in one-step nucleic acid assay-positive patients who undergo axillary dissection

| OSNA array results       | Axillary dissection after SLNB | Non-SLN metastases |  |
|--------------------------|--------------------------------|---------------------|-----|
|                          | \( n \) | %   | AvLN (min.–max.) | Present | Present | AvLN (min.–max.) | Absent | Absent | %   |
| Positive (+ and ++)      | 29/99  | 29.2| 9.46 ±0.8 (4–19) | 10/29  | 34.4    | 1.50 ±0.97 (1–4) | 19/29  | 65.5  |      |
| ITC                      | 3/29   | 10.3| 13.50 ±4.94 (10–17) | 1/3    | 33.3   | 4 (n = 1) | 2/3   | 66.6  |      |
| +                        | 12/29  | 41.3| 8.90 ±3.95 (5–16) | 4/12   | 33.3  | 1.66 ±0.57 (1–2) | 8/12  | 66.6  |      |
| ++                       | 14/29  | 48.2| 9.30 ±3.98 (4–19) | 5/14   | 35.7  | 1.00 ±0.00 (1–1) | 9/14  | 64.2  |      |

AvLN – average number of lymph nodes ± standard deviation, min.–max. – minimum–maximum.

Table V. Concordance of OSNA with histological examination – summary

| Parameter | Previous reports | Our study |
|-----------|------------------|-----------|
|           | [6] | [7] | [9] | [14] | [15] | [16] | [27] |          |
| Country   | Italy | UK  | Germany | France | Spain | NY, USA | France | Poland |
| Histological examination | Intraoperative | – | – | – | + | + | + | + |
| Postoperative | + | + | + | + | + | + | – |        |
| No. of cases/specimens | 110/131 | 203/412 | 46/80 | –/181 | 233/503 | 99/105 |
| Rate of negative nodes with OSNA | 71 |   |   |   |   | 72.38 |
| Rate of micrometastases with OSNA | 18 |   |   |   |   | 14.28 |
| Rate of macrometastases with OSNA | 11 |   |   |   |   | 13.33 |
| Sensitivity | 93.75 | 91.7 | 98.1 | 88.2 | 98.2 | 82.7 | 91.4 | 100 |
| Specificity | 83.47 | 96.9 | 91.7 | 98.4 | 94.8 | 97.7 | 93.3 | 90.47 |
| Overall concordance | 96.0 | 91.8 | 96.3 | 99.45 | 95.8 |
| Kappa | 52 |   |   |   |   | 79 |
| Predictive value | Positive | 44 | 86.8 |   |   | 72 |
| Negative | 99 | 98.1 |   |   |   | 100 |
| Discordant cases | OSNA(+)/Hist-pat (–) | 11/131 | 10/412 |   |   | 8/105 |
| OSNA(–)/Hist-pat (+) | 1/131 | 6/412 |   |   |   | 0/105 |
| Time required for OSNA | Mean | 32(0)–62(IV) | 39.6 | Not assessed |   |   |
| Min. | 22(0)–46(IV) | 26 |   |   |   |   |
| Max. | 90(IV)–97(I) | 70 |   |   |   |   |
had no metastatic non-SLNs, and theoretically they did not require ALND [19, 20]. In our study, 65% of patients who were identified in the OSNA test with SLN metastases had no metastatic non-SLNs. A study on a large series of cases could determine the cut-off point with a semi-quantitative OSNA assay to select patients who do not need additional ALND. At present, we suggest performing ALND in patients with positive OSNA qualitative and semi-quantitative results, especially in cases of macrometastases.

Sentinel lymph nodes tumor volume is considered an important factor in the prognosis of non-SLN metastasis [20]. Tumor volume in SLN is easily evaluated semi-quantitatively in OSNA, in contrast to histological examination. Micrometastases can be reliably found with OSNA because the whole SLN can be used for analysis in everyday clinical practice, which is an advantage over conventional histological examination [21]. A summary of 58 studies indicated that the presence of metastases in the axillary lymph nodes with a diameter less than 2 mm was associated with a worse prognosis [22]. The MIRROR (Micrometastases and Isolated Tumor Cells) study also indicated that both the presence of isolated tumor cells (ITC) and micrometastases in axillary lymph nodes were associated with worse prognosis in patients with early-stage breast cancer and not subjected to adjuvant systemic therapy [23].

The disadvantage of the OSNA method is homogenization of SLN and the associated lack of further possibility of histological evaluation. Thus, the same sample cannot be analyzed by both molecular OSNA analysis and subsequent postoperative histological examination [4]. Due to different parts of SLN used for analysis in each of the two methods, inconsistent results were described before between the OSNA test and histological examination [6, 15, 24]. In the study by Snook et al., a positive result of SLN in the OSNA test and negative histological examination of paraffin blocks were found in 8 of 204 patients, and the results of SLN in 5 of 204 patients were described as negative in OSNA and positive in histology [7]. The above patients either had been subjected to unnecessary ALND and potentially additional adjuvant treatment or the required ALND and adjuvant therapy had been missed. In the present study, 8 of the 105 SLN results were inconsistent between histology (negative result) and OSNA (positive result). No cases were inconsistent with a negative OSNA result and positive histology. Based on the results of our study, the overall OSNA sensitivity was 100% and specificity 90.47%, which confirms the previously described OSNA sensitivity range from 82.7% to 98.2%, and specificity from 83.47% to 98.4% (Table V) [6, 7, 9, 13, 14, 15, 25]. Based on the high concordance between histology and OSNA observed in our study, the whole SLNs in our department are now sent for molecular analysis only. The disadvantage, however, is the resulting lack of lymph node tissue for pathology examination of potential diseases other than metastasis of breast cancer [12].

The analysis of the SLN only with OSNA is associated with the risk of false negative results due to the presence of breast cancer metastases which have no CK19 expression [6]. Low CK19 protein expression in breast cancer has been described in 1.6% to 11.1% of cases, and the lack of CK19 expression was associated with the triple-negative phenotype of breast cancer (ER–, PR–, HER2–) [6, 26–28]. In a series of 197 breast cancers, Villardell et al. found no expression of CK19 in 0% to 4.2% of patients, depending on the phenotype of breast cancer [29]. Micropapillary, apocrine, mucous, medullary and mixed cancers had CK19 expression in 100% of cases after H + E staining of the whole specimen due to the focal expression of the protein. The previously described discrepancy between OSNA and histology, due to the lack of CK19 mRNA expression, was very low and ranged from 0.1% to 0.5% of lymph nodes [30]. In order to avoid false negative results, a biopsy of the primary tumor prior to surgery may include routine evaluation of CK19 expression. However, the expression of mRNA and protein may differ between the main tumor and the metastatic site. Histological examination of the one saved slice of SLN (not taken for OSNA analysis) negates this risk and, therefore, the 1 mm central part of the SLN was evaluated in our study with histology before the OSNA assay was fully validated.

In contrast, false positive OSNA results were also described and showed CK19 mRNA expression in lymph nodes from patients without cancer, e.g. with inflammation or benign epithelial inclusions [31, 32]. In addition, intramammary SLNs on lymphoscintigraphy are observed in 0.2% to 14% of patients and can theoretically cause a false positive result if the surrounding breast parenchyma has not been completely removed from the SLN before OSNA analysis [33–36]. The factor limiting the scope of this study is the lack of information on the location of SLN, and thus we cannot determine whether a higher rate of discordant cases occurred in the intramammary location of SLNs. In order to avoid the risk of error, some authors have proposed setting the cut-off point of the OSNA result above 250 copies/µl [37].

The current guidelines for cancer treatment are based on histological and not molecular criteria. However, the OSNA results proved to be more reliable than the standard histological examination of frozen sections or paraffin block and can therefore decide on further surgery and adjuvant therapy [38]. The OSNA detects nodal metastases...
of smaller size, but it is uncertain whether all patients with ITC or micrometastases require further axillary lymph node dissection, which should be the subject of further studies to establish treatment standards. When no CK19 expression in the primary tumor is observed, histological examination with H + E staining still should be recommended.

In conclusion, the OSNA analysis is a system which allows rapid and quantitative detection of mRNA CK19, which ensures high specificity with a low rate of false positives. The OSNA is a reliable, accurate tool for intraoperative diagnosis of sentinel lymph nodes in patients with breast cancer when the whole SLN is used for analysis. Therefore, OSNA minimizes the need for secondary ALND and avoids delays in the adjuvant treatment.

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Conflict of interest

The authors declare no conflict of interest.

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