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CLINICAL STUDY

Intraoperative evaluation of metastases in sentinel nodes through one-step nucleic acid amplification: Initial experience in a single institution

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ABSTRACT
The objective of this study was to gain our initial experience in one-step nucleic acid amplification (OSNA) for detecting sentinel lymph node (SLN) metastasis as compared to standard pathological staging in patients with breast cancer. Fifteen patients with preoperatively confirmed early breast cancer eligible for breast-saving therapy and sentinel lymph node biopsy (SLNB) were enrolled in the study. Lymphatic mapping and SLNs detection were performed through the magnetic method. Excised SLNs were intraoperatively examined through OSNA and frozen-section methods. All lymph nodes were postoperatively examined through histopathology and immunohistochemistry. The results of latter methods were correlated. Our initial experience proved OSNA to be a sensitive and efficient alternative to intraoperative assessment of metastases in SLN in breast cancer patients. Moreover, the information obtained by the OSNA method provides the surgeon with the possibility of assessing a more accurate prognosis during the initial surgery *(Tab. 4, Fig. 3, Ref. 36).* Text in PDF *www.elis.sk*
KEY WORDS: breast cancer, metastases, surgery, sentinel nodes, OSNA.

Introduction

Sentinel lymph node biopsy (SLNB) reduces surgical staging morbidity associated with axillary dissection while maintaining valid prognostic information (1). The primary purpose of intraoperative pathology consultation is to guide surgical management. In the case of SLNB, the information provided allows surgeons to either disclaim or immediately proceed with the axillary lymph nodes dissection (ALND) in the same surgical setting. Pathological staging is based on serial sectioning of SLNs with hematoxylin-eosin (H&E) with or without immunohistochemistry (IHC). Unfortunately, this is inadequate in an intraoperative setting. Although, frozen sections and imprint cytology are commonly used for intraoperative pathological examinations, both of them are limited by their variable accuracy and sensitivity ranging from 57 to 74 % (2–4). A failure to perform immediate consultation requires node-positive patients to return for delayed ALND. A novel test with accurate result and simple operation method is desirable.

Several molecular methods aimed at improving the detection of metastasis in lymph nodes have been developed. The one-step nucleic acid amplification (OSNA) assay is a molecular-based metastasis detection system, which uses a reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay to determine the number of mRNA copies of cytokeratin 19 (CK19) in lymph nodes. CK19 is highly expressed in approximately 98 % of breast cancers (5). Recent studies have demonstrated the applicability of the OSNA assay in the study of SLNs in various cancer sites including breast cancer (6–8). The time needed for intraoperative examination of SLNs through OSNA is comparable with that of the frozen section method. When compared to histopathological examination, OSNA offers the advantage of obtaining objective and quantitative data about the tumor load of the whole LN in a fast way (9–11). Moreover, OSNA is able to provide the surgeon with more prognostic information during the initial surgery. This has driven us to the need of gaining experience with this method in our hospital.

Materials and methods

Study design

Fifteen patients were enrolled in this pilot, single-institutional, cohort study between April and May 2022 at the Breast Unit of the 2nd Department of Gynecology and Obstetrics, Comenius University of Bratislava, Slovakia. Written informed consent was obtained

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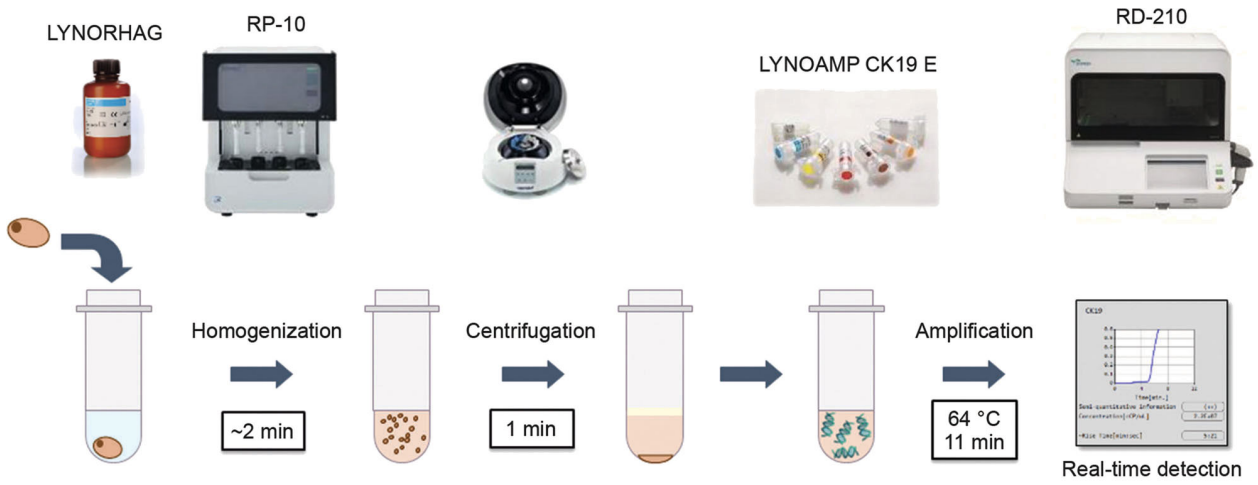

Fig. 1. Workflow of the OSNA assay.

from all patients. Included were patients with a core biopsy-proven early breast invasive carcinoma (cT1-2) and/or ductal carcinoma *in situ* (DCIS) with a high risk of invasion (e.g., high-grade DCIS with comedo necrosis) eligible for primary breast surgery. Axillary lymph nodes were preoperatively staged by clinical and ultrasound examinations. Only patients with clinically negative axillae (cN0) were involved in the study. Patients with a pacemaker or implanted device in the chest wall, iron or Magtrace allergy, as well as those pregnant or lactating were excluded. The study was approved by the Comenius University and University Hospital of Bratislava Ethics Committee.

Surgical procedures

Lymphatic mapping and sentinel lymph nodes biopsy (SLNB) were the initial surgical procedures. The day before surgery 2.0 ml of superparamagnetic iron oxide nanoparticles (Magtrace, Endomagnetics Ltd., Cambridge, UK) , were injected into the subareolar interstitial tissue. Before the axillary skin incision, magnetic count numbers from the skin were measured using the 2nd generation magnetic probe (Sentimag, Endomagnetics, Ltd., Cambridge, UK). Magnetic counts were also measured intraoperatively, *in situ*, and *ex vivo* in the SLNs, and remaining axillary nodes. SLNs were excised to the extent of lowering their counts below 10 % of their highest count or until six nodes per patient at maximum had been removed. In patients with 3 positive SLNs and more, ALND was performed. Breast-saving therapy or radical mastectomy was applied according to established criteria (12).

Evaluation procedures

Excised lymph nodes (LNs) were immediately transported to the pathology laboratory on ice (0–4 °C) to avoid RNA degradation. LNs were then halved for further evaluation. One half was examined through histopathology (frozen section and later serial sectioning with hematoxylin and eosin staining and immunohistochemistry with anticytokeratins) while the second one underwent

Tab. 1. Evaluation criteria for both methods.

Histopathology		OSNA	
Qualitative results		Quantitative results	
Macrometastases	≥ 2 mm	positive (++)	≥ 5,000 cp/µl
Micrometastases	≥ 0.2 mm	positive (+)	250–4,999 cp/µl
Isolated tumor cells	< 0.2 mm	negative (–) L	160–249 cp/µl
Without metastases	–	negative (–)	< 160 cp/µl

Tab. 2. Tumor characteristics according to the American Joint Committee on Cancer (AJCC) Staging Manual, 8th Edition, 2016.

Patient ID	Tumor type	pT stage	Grade	Molecular subtype	Pathologic prognostic stage
1	IDC-NST	pT2	2	luminal A	IIA
2	mucinous	pT2	2	luminal A	IIA
3	IDC-NST	pT1c (m)	3	luminal B-like	IA
4	IDC-NST	pT1b	3	HER2-enriched	IB
5	IDC-NST	pT2 (m)	3	HER2-enriched	IIA
6	IDC-NST	pT1c	3	luminal B-like	IIA
7	IDC-NST	pT1c	1	luminal A	IA
8	DCIS	pTis (m)	3	HER2-enriched	0
9	ILC	pT2	1	luminal A	IIB
10	mucinous	pT2	2	luminal A	IIA
11	IDC-NST	pT2	3	luminal B-like	IIB
12	IDC-NST	pT2	3	luminal A	IIB
13	IDC-NST	pT1c	1	luminal A	IA
14	IDC-NST	pT1c	3	luminal A	IA
15	IDC-NST	pT1c	2	luminal A	IA

IDC-NST = invasive ductal carcinomas of no special type, ILC = invasive lobular carcinoma, DCIS = ductal carcinoma *in situ*, HER2 = human epidermal growth factor receptor 2

Tab. 3. Comparison of OSNA assay with histopathologic examination.

Patient ID	Nr. of exscided SLNs	OSNA assay	SLN status frozen section	SLN status macro–mts	SLN status micro–mts	pN (sn) stage
1	4	–	0/4	0/4	0/4	pN0
2	3	–	0/3	0/3	0/3	pN0
3	2	–	0/2	0/2	0/2	pN0
4	3	++	0/3	0/3	1/3	pN1mi
5	6	–	0/6	0/6	0/6	pN0
6	3	++	1/3	1/3	1/3	pN1a
7	6	–	0/6	0/6	0/6	pN0
8	3	–	0/3	0/3	0/3	pN0
9	5	++	3/5	3/5	0/5	pN1a
10	4	–	0/4	0/4	0/4	pN0
11	4	++	2/4	2/4	0/4	pN1a
12	4	+	1/4	1/4	1/4	pN1a
13	2	–	0/2	0/2	0/2	pN0
14	2	–	0/2	0/2	0/2	pN0
15	2	–	0/2	0/2	0/2	pN0

SLN status = number of positive sentinel lymph nodes/number of examinated sentinel lymph nodes, OSNA assay = results the one-step nucleic acid amplification assay for the detection of SLN metastases (negative (–), micrometastases (+) and macrometastases (++), macro-mts = macrometastases, micro-mts = micrometastases

the OSNA assay. Lymph nodes were homogenized in 4 mL of LYNORHAG lysis buffer (Sysmex Corporation, Kobe, Japan). CK19 mRNA was amplified by reverse-transcription loop-mediated amplification (RT-LAMP). A 2 µL aliquot was used for automated real-time amplification of CK19 mRNA with the ready-to-use LYNOAMP reagent on the RD-100i analyzer (Sysmex Corporation, Kobe, Japan) (Fig. 1). The OSNA assay requires a time span of approximately 30 to 40 minutes from start to completion, which almost equals to that required for conventional pathology tests. For OSNA analysis, a standard positive control sample containing 5,000 copies of CK19 mRNA/µL (cp/µl) , and negative control sample containing 0 cp/µL were used for calibration in each study. The results were expressed as a level of CK19 mRNA. Based on the cut-off values determined for the OSNA assay, values < 250 cp/µL of CK19 mRNA were considered negative (–) ; values between 250 and 4,999 cp/µL were considered as micrometastases (+) ; and values ≥ 5000 cp/µL were considered as macrometastases (++). The total tumor load (TTL) is defined as the sum of the CK19 mRNA copies from all positive SLNs of the patient. Evaluation criteria for both methods are shown in Table 1.

The frozen halves of SLNs were intraoperatively examined with hematoxylin-eosin staining and postoperatively with immunohistochemistry (IHC). For IHC, the monoclonal mouse anti-human cytokeratin antibody (clones AE1/AE3, code M3515, DAKO) was used. According to established histopathologic criteria (12) , lymph node macrometastases measured > 2 mm; micrometastases were defined as a focus of metastasis measuring > 0.2 mm and ≤ 2 mm (pN1mi). Isolated tumor cells were defined as microscopic clusters and single cells measuring 0.2 mm or less (pN0i+). Immunoreactivity for human epidermal growth factor receptor 2 (HER2) , estrogen receptors (ER) , and progesterone receptors (PR) were studied by means of the VENTANA- BenchMark-XT computerized automated system, using the *ultra*View Universal DAB Detection Kit (ROCHE) which detects specific mouse and rabbit primary antibodies bound to an antigen in paraffin-embedded tissue sections. The intensity score for HER2 was defined in scale of 0 to 3 (for absent, weak, moderate or strong staining, respectively). In cases with HER2 score 2+ (equivocal) , an additional evaluation through silver DNA *in situ* hybridization (SISH) was used for ultimate positive or negative results. Histological grade of tumors was evaluated by the Elston-Bloom and Richardson systems (12). According to histopathology results, the tumors were categorized as to intrinsic subtypes and pathologic prognostic stage groups as seen in Table 2.

Statistical analysis

The SLN metastases detection rate of OSNA assay and histopathology was compared using the χ^2 test. The differences in concordance rates of detected SLN metastases, and measurements of disease burden in involved SLNs between OSNA and histopathology were evaluated.

Results

The mean age of our group of patients was 60.1 years (range 41–76 years). Tumor characteristics of all surgically treated breast lesions are shown in Table 2. Infiltrating ductal carcinoma of no special type (IDC-NST) was the most common tumor; in two patients it was of mucinous subtype. One patient was recruited with high-grade multicentric ductal carcinoma *in situ* (DCIS) and another one presented with infiltrating lobular carcinoma. Breast-saving therapy was performed in almost all patients, only two of them being submitted to radical mastectomy. All tumors were removed with safe surgical margins through the first operation and no additional re-excision of margins was needed.

A total of 54 SLNs were examined through both methods in this group of patients. The mean nodal detection rate (the proportion of SLNs detected per patient) was 3.4 (from 2 to 6). Nine patients presented with negative SLNs in frozen section confirmed in postoperative paraffin serial sections, which was concordant with examination through OSNA (OSNA -). In one patient (No. 7) , the OSNA assay value of 180 cp/µL of CK19 mRNA in one of the six excised SLNs was considered negative although this value would indicate that isolated tumor cells (ITC) had been involved. No micrometastases < 0.2 mm or ITC were found in this SLN in postoperative histology. In four patients with 1 to 2 positive SLNs, results of both methods (OSNA and histology) were concordant. One of our patients (No. 9) presented metastases in three of five examined SLNs, and additional axillary dissection

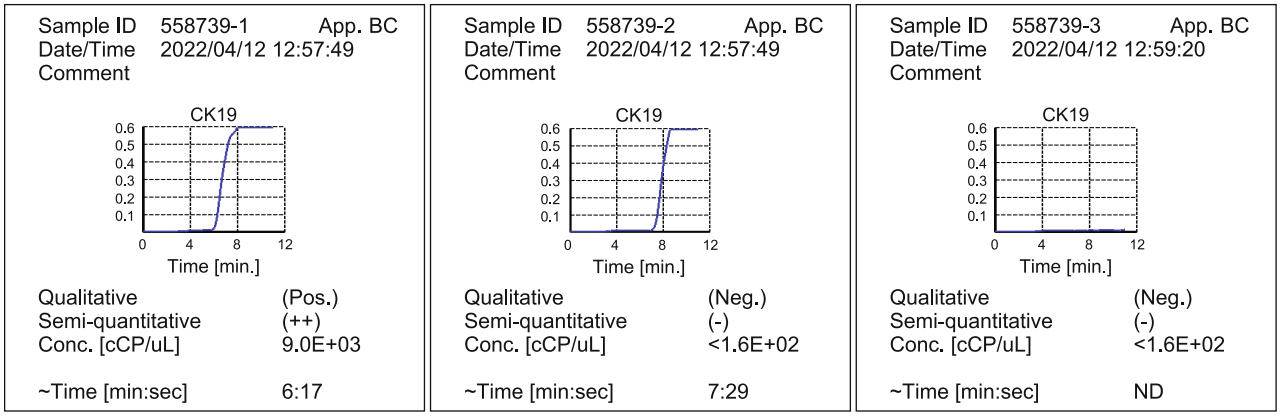

Fig. 2. Patient No.4: results of the intraoperative OSNA analysis of all three SLNs. Positive results of SLN1 with 9,000 copies of CK19 mRNA/µL (on the left) suggest that in this half of the node, there was a greater total tumor load and therefore, the test result was OSNA-positive (++).

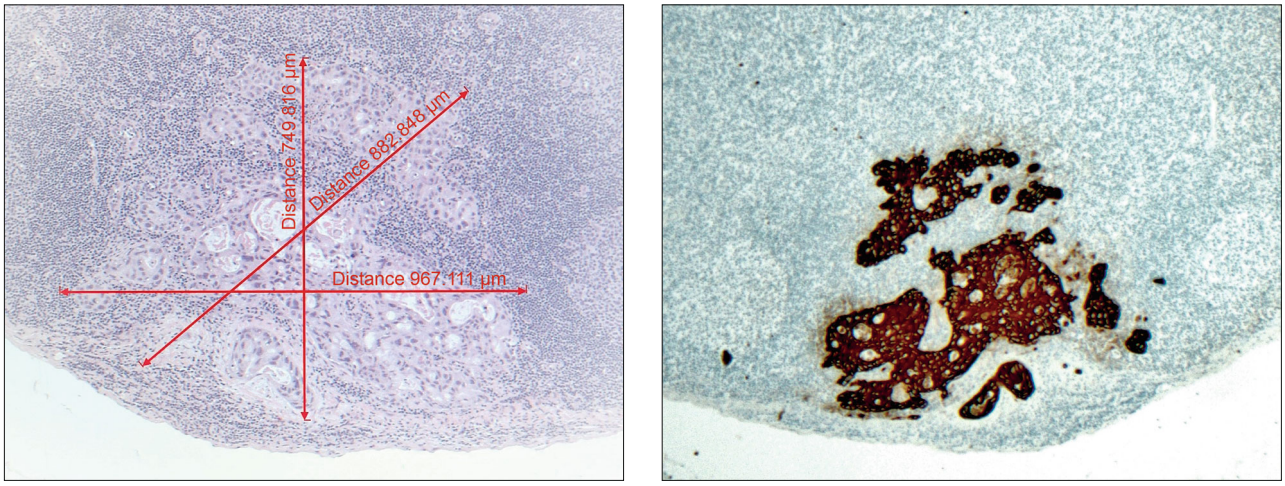

Fig. 3. Patient No.4: postoperative paraffin sections of SLN1. Micrometastases ≤ 1 mm, H&E, x10 (on the left) and immunohistochemistry with anticytokeratins, x10 (on the right).

was provided. No further metastases in 17 axillary non-SLNs were found. The comparison of the OSNA assay with the histopathologic examination is shown in Table 3. The only discordant result could be found in patient No. 4, in whom intraoperative frozen-section examination showed negative results in 3 SLNs but the OSNA assay was positive with 9,000 cp/µl (++) in the first excised SLN. The qualitative and quantitative results of the real-time detection of TTL in all 3 SLNs from this patient are presented in Figure 2. The second and third SLNs were negative by means of both methods. The postoperative evaluation of SLNs in paraffin sections showed micrometastases of 1 mm in the first SLN (Fig. 3). We assume that it was the unevenness in allocation of metastases in the slices of lymph SLNs that caused the discordance in OSNA and histology results (see also below in Discussion). The comparison of the OSNA assay with the histopathologic examination is shown in Table 3.

Discussion

Axillary nodal staging is now limited exclusively to the study of SLNs, thus preventing the determination of the real axillary lymph node status. It is expected that ALND can be gradually more frequently avoided in the future in patients with SLN-positive breast cancer with favorable tumor characteristics. The ACOSOG Z0011 trial has proposed that ALND can be avoided in breast cancer patients with clinical cT1-T2 cN0 invasive breast cancer, 1 to 2 positive SLNs containing metastases, undergoing breast conserving surgery and adjuvant tangential whole-breast irradiation (13, 14). The purpose of intraoperative pathology consultation is to guide surgical management according to the results of SLNs examination. False-negative intraoperative frozen section results of SLNs have been reported, in particular after neoadjuvant chemotherapy (15, 16). In 2016, we published results from our retrospective analysis, where clinical and pathologic data from 163

patients who underwent SLN biopsy followed by ALND were collected (18). Metastases in SLN were present in 67 (41 %) of patients, while 48 patients (29.4 %) had metastases also in non-SLN. Some studies have described predictive models for axillary lymph node involvement (defined as the presence of micro- or macrometastases) in four and/or more nodes (17, 18). The proportion of disease-free patients at 5 years after diagnosis can range from 96 % in patients with no axillary involvement to 66 % in those with involvement of over 4 lymph nodes (19).

In their study, Peg et al (20) could show that the OSNA evaluation of SLNs can classify patients as being at high or low risk based on the tumor burden, thus making this a new tool for customizing patient treatment. Nomogram was capable of classifying 63.5 % of patients as a low-risk group with only a 3.1 % chance of having metastases in ≥ 4 ALNs. (i.e., for decisions about regional radiotherapy when ALND is not performed). Nineteen studies were included in the meta-analysis of OSNA results in the evaluation of SLNs in breast cancer patients published recently by Shi et al (21). This meta-analysis was conducted with the aim to assess the diagnostic performance of OSNA for SLN overall metastases and macrometastases in breast cancer with the latest papers. The pooled sensitivity (0.85) and specificity (0.98) were showing that the macrometastasis judgments by means of OSNA were in excellent accordance with pathological judgments. A high concordance rate of over 85 % suggests that OSNA could be an alternative technique to histopathological examination in terms of its ability to detect LN metastases. Cuadras et al (22) recently published the latest evidence related to the detection of LN metastases in several tumors by using OSNA compared with the conventional H&E method. They analyzed results from 25 studies from six different groups of tumors: breast, gastrointestinal, gynecological, lung, head and neck and prostate cancers. When compared with the H&E method, the reported results showed high specificity, concordance rate, and negative predictive value of OSNA assay, specifically, in breast cancer patients, in whom high negative predictive value provides enough evidence to become the standard for SLN evaluation. In 2022, Osako et al (23) published a prediction model for early systemic recurrence in breast cancer using OSNA analysis of SLNs in a large-scale, multicenter cohort study. SLNBs from 4,757 patients with breast cancer were analyzed. The SLN tumor burden was an independent prognostic factor for early breast cancer. in their recently published study, Hintzen et al (24) referred that OSNA did not lead to overtreatment in the current era of axillary management.

In triple-negative and HER2-positive tumors > 2 cm and/or positive axillary lymph nodes, a neoadjuvant (preoperative) systemic treatment (NST) should be preferred (12). The high rate of nodal downstaging leads to the development of four multi-institutional trials which demonstrated that SLNB reliably stages the axilla in women who are initially node-positive and become clinically node-negative after NST, provided that more than two SLNs are obtained (25, 26). The amount of residual disease in lymph nodes after NST remains an important prognostic factor. The data show that the rate of positive non-SLN is high independent of the size of the SLN metastasis, and any size metastasis should be considered significant, i.e., in contrast to the non-NST setting where the size of SLN metastases is not correlated with the rate of positive non-SLN (27). False-negative intraoperative frozen section results of SLN range from 8 % to 14.2 % (25, 26). These rates can be improved by marking the biopsied positive node (s) with metal clips to verify their removal through targeted axillary dissection (27). Identification of any tumor deposits in post-NST SLNB prompts ALND (26, 27). In the NEOVATTL study (28) , high OSNA TTL (> 25,000 cp/μL) after NST increased the risk of disease recurrence three-fold as compared with low TTL after NST.

It seems that the OSNA method will find its application also in the ultrastaging of SLNs in endometrial and cervical cancers. Raffone et al (32) analyzed four studies with 237 patients and 691 lymph nodes. OSNA showed sensitivity of 0.88, specificity of 0.93, and high diagnostic accuracy (AUC = 0.959). Recently, a preliminary report on evaluation of the accuracy of the OSNA assay in diagnosing LN metastasis of uterine cancer has been published (33).The concordance rate between histological examination and OSNA assay in cervical cancer and endometrial cancer was 95.9 % and 95.2 %, respectively. In cervical cancer, the sensitivity, specificity, and negative predictive value of the OSNA assay were 80 %, 97.7 %, and 97.7 %, respectively.

This study represents our initial experience with OSNA in terms of its intraoperative evaluation of metastases in SLNs in a small group of patients with early breast cancer. Our study has some limitations. In this type of comparative study between two techniques from a single SLN, it is not possible to use exactly the same tissue for both tests. Especially in the case of low-volume metastases, there is the possibility that metastatic cells are located only in the part of tissue studied by means of OSNA while not being present in that analyzed by histopathology. This phenomenon was described as a “tissue allocation bias” (6). We assume that in patient No. 4, it was the unevenness in allocation of metastases in the slices of lymph nodes that have caused the discordance between OSNA and histology results. The concentration of 9,000 copies of CK19 mRNA/μL suggests that in this half of the node, there was a greater total tumor load and the test result was therefore OSNA-positive (++). The post-operative evaluation of the node in formalin-fixed tissue sections showed micrometastases of 1 mm (Figure 3). The ability of the OSNA assay to detect small-volume metastases demonstrates a greater sensitivity of the molecular analysis as compared with pathological staging. In addition, pathologic examination inspects only a limited portion of SLN on a slide specimen, i.e., a macrometastasis may be falsely identified as micrometastasis. As to the objective and quantitative outcome as well as automated technique, OSNA assay has an obvious superiority over traditional intra-operative SLNB such as the frozen-section method. When compared to histopathological examination, OSNA offers the advantage of obtaining objective and quantitative data about the tumor load of the whole LN in a fast way. A potential disadvantage of examining the whole LN with OSNA is that there is no tissue left for subsequent histopathological examination following complete homogenization. Nevertheless, it is still possible to use OSNA lysate for RNA-based molecular tests, thus allowing for any follow-up molecular testing (36).

Tab. 4. OSNA molecular cut-offs for clinical decision-making.

100,000 cp/µl	**Prediction** Risk of having ≥ 4 positive lymph nodes
25,000 cp/µl	**Prognosis** Classification of patients in low or high risk
15,000 cp/µl	**Prediction** Risk of non-SLN involvement
5,000 cp/µl	**Size of metastasis** Micro- or macrometastasis
250 cp/µl	**Presence or absence of metastasis**
< 250 cp/µl	**Prognosis** De-escalation of therapy in ER+/HER2- OSNA negative

Conclusions

OSNA can assess the occurrence of metastasis in the whole lymph node and estimate the total volume of tumor cells quantitatively. This is one important advantage of OSNA over pathological assessment, and it enables a more reliable prediction of prognosis. Our initial experience verified OSNA as a sensitive and efficient alternative to pathological evaluation for the assessment of SLNB in breast cancer, even on a small number of patients. Using OSNA for intraoperative SLN analysis avoids second surgery for ALND in most breast cancer patients. In neoadjuvant setting it can predict not only the presence of metastases in non-SLNs but also the disease-free survival of patients. OSNA molecular cut-offs for clinical decision-making are shown in Table 4. This molecular assay provides fast and reliable results and has already been successfully incorporated in the standard treatment guidelines for many cancer sites such as colon, stomach, prostate, uterus, lung and others.

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