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ORIGINAL PAPER

Intra-operative use of one-step nucleic acid amplification (OSNA) for detection of the tumor load of sentinel lymph nodes in breast cancer patients

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Abstract
Background The purpose of this single-center study was to determine the practicability of the intra-operative use of one-step nucleic acid amplification (OSNA) as the only method for detection of SLN. The OSNA system has been well described and is supposed to be as accurate as conventional histology.
Methods Three hundred and thirty SLNs from 143 breast cancer patients were analyzed in an intra-operative setting. The CK19-copy number was determined by OSNA and divided into 3 results ("−" no metastasis; "+" micrometastasis; "++" marcometastasis). If OSNA gave a positive result, an axillary lymph node dissection was carried out during the same session. The central 1-mm slice of each node was obtained for permanent histology. Additionally, the results were correlated to clinicopathological factors, and the time for the intra-operative use was evaluated.
Results Thirty-nine of the 143 patients were OSNA positive, 22 with macrometastatic and 17 with micrometastatic spread. The mean time for the OSNA run with one SLN was 34.4 min. We could show a correlation between the tumor size and OSNA positivity as well as between the numbers of OSNA positive SLNs with the tumor load of associated non-SLNs. Furthermore, we found that a cutoff CK19 copy number of 7,900/μL indicates a positive non-SLN result with the highest sensitivity (91 %) and specificity (61 %).
Conclusion We found OSNA to be very helpful for the intra-operative determination of the tumor load of a SLN as a basis for decision-making concerning further surgical axillary intervention. OSNA allows precise differentiation of micro- from macrometastasis, and the CK19 copy number predicts the probability of tumor load in other axillary lymph nodes and might help to find adequate adjuvant treatment options. This objective method is well suitable for everyday use and may reduce the pathologic workload and the risk of secondary operative interventions with all associated costs and stress for the patients.

Keywords Carcinoma · Breast cancer · Sentinel lymph node/SLN · Nucleic acid amplification/OSNA · Tumor load · CK19

Introduction

The detection of sentinel lymph node (SLN) metastases in breast cancer patients is conventionally determined by intra-operative histopathological examination of frozen sections or touch imprints. The application of these methods, however, is limited due to their rather low sensitivity, especially with respect to detection of micrometastases and invasive lobular cancer (Van de Vrande et al. 2009; Tew et al. 2005; Motumura et al. 2000). In addition, the preparation of frozen sections can lead to tissue loss (Layfield et al. 2011).

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Post-operative examination of permanent sections might then detect a positive SLN and lead to a second surgery with complete axillary dissection. This scenario can have an influence on both the psychological and physical condition of the patient as a second operation is associated with extra emotional stress, is more demanding on an operative level for the surgeon (Goyal et al. 2008) and might delay a necessary adjuvant treatment (Klingler et al. 2013). Besides this, extra bed and hospital time is a disadvantage from an economic point of view (Cutress et al. 2010).

Intense postoperative histology including serial sectioning and the use of immunohistochemistry can lead to an upstaging rate of 28 % (Park et al. 2009). However, the use of paraffin histology is hampered by a variety of different protocols applied throughout clinics and breast cancer units (Cserni et al. 2004) and interobserver disparities in interpreting the results (Roberts et al. 2003).

Upstaging can also occur by applying molecular methods, and the detection of micrometastatic disease in SLNs of breast cancer patients via RT-PCR correlates with prognostic factors (Gimbergues et al. 2007; Gillanders et al. 2004). As RNA isolation is a prerequisite to RT-PCR, this approach usually takes too long for intra-operative purposes. A commercially available intra-operative molecular diagnostic tool based on one-step nucleic acid amplification (OSNA) using automated measurement of cytokeratin 19 (CK19) mRNA has been developed and was successfully evaluated at our hospital (Schem et al. 2009) and others (Tsujimoto et al. 2007; Visser et al. 2008; Tamaki et al. 2009; Snook et al. 2011). Test results are available after 30–40 min with a ready to use reagent kit (Sysmex, Kobe, Japan).

We evaluated this molecular method for the detection of SLN metastases in 143 breast cancer patients during intra-operative analysis of SLNs within the operating theater. In addition, results based on OSNA were correlated to clinicopathological factors.

Materials and methods

Patients and tissue handling

Both the preparation of the SLN and OSNA analysis were performed intra-operatively in a laboratory within the operating theater complex by a laboratory technician, with no pathologist involved. Three hundred and thirty SLNs from 143 breast cancer patients were analyzed.

Upon arrival, the SLN was cleaned from surrounding fat, and the central 1-mm slice was obtained with a predesigned cutter and reserved for permanent histology.

The remaining lymph node tissue was shortly homogenized with 4 mL of a lysis buffer solution, centrifuged twice and processed according to the manufacturer's instructions. Two microliter of the supernatant was analyzed with the RD-100i (Sysmex, Kobe, Japan) for CK19 mRNA amplification. A positive and negative CK19 mRNA control as provided in the Lynoamp reagent kit (Sysmex) was included in each sample run. OSNA results were categorized in (−) if <250 CK19 mRNA copies/μL were present, (+) for samples with a CK19 mRNA copy number 250–5,000, and (++) with SLNs contained more than 5,000 CK19 mRNA copies/μL (Tsujimoto et al. 2007).

If OSNA gave a positive result (++, equivalent to a macrometastasis; +, equivalent to a micrometastasis), axillary lymph node dissection was carried out during the same surgical session. In OSNA positive cases, one hematoxylin & eosin (H&E) section was prepared postoperatively. If OSNA was negative, H&E staining was performed every 200 μm of the 1-mm slice, CK19-staining was affiliated in discrepant results as well as all non-SLNs were pathologically examined after the operation. In case histology was positive, a secondary operation was assigned.

Statistics

Quantitative values were presented as mean and standard deviation, minimum and maximum, as well as quartiles. They were tested for normal distribution using the Kolmogorov–Smirnov test; in case of small case numbers, the Shapiro–Wilk test was applied. Because of significant deviations from normal distribution, two independent samples were compared using the Mann–Whitney U test.

Ordinal and nominal scaled values were displayed in absolute and percent frequencies. Two of each of these values were compared in contingency tables and tested for dependence with the χ^2 test. If the expected frequencies turned out to be too small, the exact Fisher test was used.

Furthermore, a ROC-analysis was used to find a CK19 mRNA copy number, which represents a cutoff to distinguish between positive and negative non-SLNs; for this, the premise was maximal sensitivity and maximal specificity.

The tests were two sided with a significance level of 5 %. An alpha adjustment for multiple testing was not applied, and the results were interpreted accordingly. Statistical calculations were done with PASW 18 (SPSS Inc., IBM, Chicago, IL, USA).

Results

In this single-center study, 330 SLNs from 143 breast cancer patients were analyzed by OSNA in an intra-operative setting. Patients and tumor characteristics as shown in Table 1 reflect an average distribution among the local population; the medical treatment indication was set

according to the Guidelines of the German Society of Gynaecology and Obstetrics (DGGG) and Senology.

The time, from arrival of the SLN to completion of the OSNA run, was 34.4 min for one SLN and 40.4 min for two SLNs (Table 2). The mean number of SLNs examined in one patient was 2.51.

During intra-operative OSNA use, 39 patients were positive and 104 were negative in OSNA. These results were confirmed by permanent histology of the 1-mm central slice. Twenty-eight of these patients were OSNA positive but histologically negative; 2 patients were histologically positive but negative in OSNA. In total, 41 axillary dissections were carried out. Macrometastatic spread (OSNA ++ results) in the examined lymph nodes was found only in 22 patients, micrometastasis in 17 patients (OSNA + results).

Furthermore, the correlation between SLN metastatic status as determined by OSNA and breast cancer prognostic factors was evaluated. A significant association between a positive OSNA result and tumor size was found (Table 3). A positive OSNA result in the SLN was observed more often in breast cancer patients with a tumor larger than 2 cm as opposed to patients with tumors smaller than 2 cm ($P = 0.039$). The number of OSNA positive SLNs is also predictive for the tumor load in non-SLNs (χ^2 test for linear trend, exact, $P = 0.027$) (Table 4). The higher the number of metastatic SLNs, the higher the probability for non-SLN metastases. Moreover, the tumor load in a SLN is also of great importance. The CK19 copy number, as indicated by (++) versus (+), is a predictive factor for non-SLN positivity (Table 4). A significant association between the two factors was seen (Fisher's exact test, $P = 0.011$). When an (++) OSNA result was obtained for one SLN, it was 13 times more likely that a corresponding non-SLN was positive as compared to a (+) result [odds ratio, OR = 13.3, 95 % CI = (1.5; 118.9)]. With a sensitivity of 90.9 % and a specificity of 60 %, a CK19 copy number of 7,900/μL or above in one SLN will indicate a tumor positive non-SLN (maximal Youden Index) (Table 5; Fig. 1).

Table 1 Patient characteristics (N = 143 patients)

Characteristics	Number of patients
Median age (range)	61 (26–87)
Histological type	
Invasive ductal carcinoma	101
Invasive lobular carcinoma	35
Other	7
Tumor size	
pTis	2
pT1a	4
pT1b	37
pT1c	65
pT2	29
pT3	6
Estrogen receptor status	
Positive	133
Negative	10
Progesterone receptor status	
Positive	120
Negative	23
HER2/neu expression	
Positive	6
Negative	137
Vascular invasion	
V0	142
V1	1
Lymphatic invasion	
L0	135
L1	8
Menopausal status	
Premenopausal	32
Postmenopausal	106
Perimenopausal	5
Number of SLN removed	
1	32
2	48
3	31
4	25
5	6
8	1

Discussion

The reliability of the OSNA method has been described extensively and already been examined in our hospital (Schem et al. 2009). In 2011, a large multicenter trial was published by Feldman et al. affirming this method to be at least comparable to conventional histology and immunohistology in terms of sensitivity of the detection of metastatic carcinoma in lymph nodes. Le Frere-Belda et al. (2012) lately stated a sensitivity of 91.4 % with a

Table 2 Time for intra-operative OSNA use in minutes

	1 SLN	2 SLN	3 SLN	4 SLN
Range	31–40	37–47	38–55	41–63
Mean	34.4	40.4	46.9	52.1
SD	2.5	2.3	4.3	4.2
CV (%)	7.3	5.7	9.1	8.0

SD standard deviation, *CV* coefficient of variation

Table 3 Prognostic factors and OSNA results

Prognostic factor	OSNA result			*P* value
	− $N = 102$	+ $N = 17$	++ $N = 22$	
Tumor size (cm)				
≤2	81	14	12	0.038
>2	21	3	10	
Histopathological type				
Invasive ductal	75	13	13	0.604
Invasive lobular	23	4	8	
Other	4	0	1	
Estrogen receptor status				
Negative	7	2	1	0.750
Positive	95	15	21	
Progesterone receptor status				
Negative	18	2	3	0.875
Positive	84	15	19	
HER2/neu status				
Negative	97	17	21	1.000
Positive	5	0	1	
Vascular invasion				
V0	101	17	22	1.000
V1	1	0	0	
Lymphatic invasion				
L0	97	17	19	0.164
L1	5	0	3	
Menopausal status				
Premenopausal	21	2	9	0.017
Postmenopausal	80	12	12	
Perimenopausal	1	2	1	

specificity of 93.3 % for detecting metastases on the OSNA basis. We present a prospective single-center study at an academic hospital evaluating the practicability of an intra-operative application of OSNA. Unlike other published studies, we used OSNA as the only method to determine a possible metastatic tumor load of a SLN in an intra-operative setting consequently leading to an axillary dissection in the same session in case of OSNA positivity. Of the 143 patients in this study, 11 patients were positive in OSNA as well as in histological staging. Twenty-eight patients were positive in OSNA but negative in histological examination. This seems to be reasonably as about 90 % of the lymph node tissue was referred to OSNA. Two patients were found to be histologically positive and OSNA negative, which might be explained by the fact that there is a low percentage of CK 19-negative carcinomas of the breast (Chu and Weiss 2002), causing a tissue allocation bias. Noteworthy is also the description of a low expression of CK19 in triple-negative carcinomas (Parikh et al. 2008). To avoid false negative results, the initial diagnostic biopsy sample of the tumor could be tested on CK 19 before using OSNA as the only method as proposed by Vilardell et al. (2012). On the other hand, single cases of false-positive OSNA results are described on pathological entities such as cystic benign lesions and ectopic breast tissue, but are extremely rare (Bernet et al. 2011). A certain physiological expression of CK 19 in the surrounding epithelia incorrectly examined with the SLN-tissue does not lead to a positive OSNA result as there is a lower assay cutoff at 250 copies/μL. Considering the fact that also isolated tumor cells are not represented in the determined margins of this assay, Le Frère-Belda et al. (2012) suggested a new lower cutoff at 380 copies/μL for positivity, without changing the patient's classification in their study.

Table 4 OSNA positive SLN versus non-SLN positivity

OSNA positive SLN	Histology negative non-SLN	Histology positive non-SLN	Positive non-SLN/positive SLN (%)	*P* value
+ Versus ++				
+	16	1	5.9	0.011[a]
++	12	10	45.5	
No. of positive SLNs				
1	20	4	17.4	0.027[b]
2	8	5	38.5	
>2	0	2	100	

[a] Fisher's exact test, [b] χ^2 test for linear trend (exact)

In our collective, a tumor size of more than 2 cm indicated a higher probability of OSNA positive SLNs. Additionally, the number of OSNA positive SLNs and the tumor load in them correlated with the amount of metastasis in corresponding non-SLNs. Besides that, the use of OSNA enables a reliable intra-operative differentiation of macro- and micrometastases. This plays an important role as we could show that further axillary metastasis is 13 times more likely in (++) compared to (+) OSNA positive SLNs, which is consistent with the data of Le Frère-Belda et al. (2012) as well as Peg et al. (2013). In the next step, we examined whether it is possible to assist the surgeon's decision concerning further axillary intervention by evaluating the amount of metastatic tumor load in a SLN. According to our analysis, a reasonable cutoff for the tumor load in one SLN predicting metastasis in further axillary lymph nodes should be set at 7.900 copies/μL, demonstrating the highest sensitivity (91 %) and specificity (61 %) in our collective. These findings match the recently published data of Ohi et al. (2012), Espinosa-Bravo et al. (2013) and Peg et al. (2013). Their groups also evaluated a correlating amount of total tumoral load (TTL) in a SLN with slightly different margins, partially depending on the hormonal status of the primary tumor.

Table 5 Cutoff for the CK19 copy number indicating non-SLN positivity with the highest sensitivity and specificity (ROC curve analysis, $P = 0.016$)

Positive if bigger or equal to	Sensitivity	1-Specificity	Specificity	Youden index
1,450	1.0000	0.5714	0.4286	0.4286
1,600	0.9091	0.5714	0.4286	0.3377
1,800	0.9091	0.5357	0.4643	0.3734
2,050	0.9091	0.5000	0.5000	0.4091
2,550	0.9091	0.4643	0.5357	0.4448
4,350	0.9091	0.4286	0.5714	0.4805
7,900	**0.9091**	0.3929	**0.6071**	0.5162
11,000	0.7273	0.3929	0.6071	0.3344
12,500	0.7273	0.3571	0.6429	0.3701
13,500	0.6364	0.3571	0.6429	0.2792
16,500	0.6364	0.3214	0.6786	0.3149
20,000	0.5455	0.3214	0.6786	0.2240
22,500	0.4545	0.2857	0.7143	0.1688
1,450	1.0000	0.5714	0.4286	0.4286

Bold values were calculated by ROC-analysis

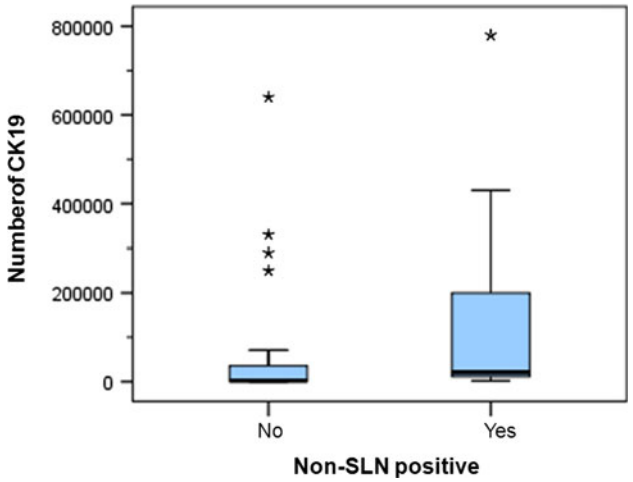

Fig. 1 Non-SLN positivity in relation to SLN CK19 copy number/µL

Complete axillary dissection to determine the exact lymph node status leads to greater costs for hospital and health systems as well as higher morbidity and psychological stress for the patient than SLN biopsy alone and should therefore be avoided whenever possible (Goyal et al. 2008; Cutress et al. 2010). More than ever in times where the axillary dissection as a consequence of a positive SLN is discussed very controversially, the objective measurement of the CK-19-copy number in a SLN may assist to this decision especially in an intra-operative setting. Data shown by Giuliano raise the question whether patients actually do benefit from axillary dissection in early tumor stages, even in cases with up to two metastatic SLNs involved (Olson et al. 2008). Up to now, this largest trial showed no significant differences concerning overall survival, disease-free survival and local recurrence between patients undergoing SLN biopsy alone and patients with SLN biopsy followed by axillary dissection (ACOSOG Z0011). But even if the intra-operative examination loses relevance in this specific cohort, the question still remains essential for all patients with tumors exceeding 5 cm in diameter or DCIS as well as for all non-breast-conserving-therapies. Furthermore, the MIRROR-Study from the Netherlands was published in 2012 showing a reduced 5-year DFS in breast cancer patients with isolated tumor cells (77 %) and micrometastases (77 %) compared to a pN0-status (85 %) (Vestjens et al. 2012). Interestingly, this group stated a changing in the N-classification after central pathological review in 24 % of their patients (with an upstaging in 18 % of the patients). The problem of interobserver variability in the pathological histological examination was addressed already in 2003 (Roberts et al. 2003). At the time of this study, the Guidelines of the German Society of Gynaecology and Obstetrics (DGGG) and Senology recommended the complete axillary dissection after detection of micro- and macrometastasis in a SLN. For 2012, in due consideration of the data shown by Guiliano and the clinical experiences, the German Guidelines have been adjusted. In case of micrometastatic tumor load in a SLN, an axillary dissection can be resigned. Therefore, an observer independent tool, which could differentiate micro- from macrometastasis in a lymph node in an intra-operative setting, is needed more than ever and might be found in molecular assays. In this context, also a major disadvantage of the OSNA method has to be mentioned. With the loss of a pathological examination, the microscopic inspection of a metastatic lymph node concerning a possible extracapsular tumor growth is impossible, which thus might have an impact on the further adjuvant treatment decisions.

In consideration of the huge workload for pathologists and the associated costs, the discussion of the practicability

of OSNA might also be led from an economic point of view. Especially, the secondary operations with de novo hospital treatment strain the health care systems. Although the setting of the study is not strictly comparable to the treatment setting of other trials, Guillén-Paredes et al. (2011) found a saving of about 440€ per patient using OSNA instead of conventional postoperative histology. Whether there is a discrepancy concerning the costs of OSNA and histology in an intra-operative setting remains to be evaluated. In our study, the mean time of an OSNA run was 34.4 min for one and 40.4 min for two SLNs. This seems to be consistent with other studies but is slightly longer than the time adjusted by the pathologists in our hospital; however, it is well suitable for an intra-operative application.

In summary, we examined the practicability of the use of OSNA for the detection of the tumor load of a SLN for the intra-operative decision-making concerning the need of an axillary dissection in 143 breast cancer patients. Besides the clinicopathological factors of the primary breast cancer, which are generally taken into consideration before the surgical intervention, the application of OSNA offers excellent possibilities for the detection of number of positive SLNs, a very accurate differentiation of micro- from macrometastatic tumor spread and the determination of the exact tumor load in a SLN. We could show that these factors are related to the probability of further metastatic spread in associated non-SLNs and might support the surgeon's oncological decision concerning the need of further axillary intervention.

As OSNA provides an opportunity for a fast, objective processing of the SLN, free from the need of pathologic workload, OSNA should be seen as a valuable method for intra-operative detection and differentiation of metastases in SLN. Further studies will be necessary to confirm the discussed data and to set the exact margins for the total tumor load in a SLN indicating further axillary metastatic spread.

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