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Diagnosis of the sentinel lymph node in breast cancer: a reproducible molecular method: a multicentric Spanish study

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Aims: Standardization of the sentinel node (SN) as a diagnostic tool has not yet been achieved, because the protocol for histopathological study is highly variable between centres. We compared the results of a new method with conventional histological tests and evaluated its feasibility for intra-operative evaluation, and propose it as a method to standardize the sentinel node evaluation procedure.

Methods and results: Trial 1 included 181 cases; in parallel, 2-mm-thick sections of the SN were processed alternately for histological analysis and for the one-step nucleic acid amplification (OSNA) procedure. A final concordance of 99.45% was observed in the first trial of our study. For trial 2, the timing of every procedural step was recorded in an electronic database in order to discern the time spent for each step, the total SN evaluation time and to identify areas of improvement. In the second trial, after a learning period and feedback on data recorded, we spent a mean of 31 min for the entire SN evaluation procedure.

Conclusion: Our multi-centric trial using the OSNA assay for sentinel node evaluation in breast cancer demonstrates that this is a highly sensitive, specific and reproducible technique that allows for standardization of the SN diagnostic procedure, a necessary, and until now unresolved, issue.

Keywords: breast cancer sentinel node, molecular diagnosis, OSNA

Abbreviations: DCIS, ductal carcinoma *in situ*; ITC, isolated tumour cell; LN, lymph node; OSNA, one-step nucleic acid amplification; SN, sentinel node

Introduction

During the past 15 years, histopathological study of the sentinel node (SN) has been proposed as the standard procedure for conservative axillary lymph node (LN) surgery in breast cancer patients.[1] However, adequate diagnostic standardization has not yet been achieved and protocols for histopathological evaluation are highly variable between centres. Different intra-operative diagnostic procedures for sentinel node assessment include touch imprint of one or more slices, one or several intra-operative frozen sections stained

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with haematoxylin and eosin (H&E), with added immunohistochemistry as the most exhaustive method.[2] The highest reported false negative (FN) rate occurs with the touch imprint method, followed by evaluation using a non-exhaustive histological method. The immunohistochemistry method improves the diagnostic results.[2] Despite this, as there is no standardization of the actual histopathological procedures, there is great difficulty in comparing results between centres, particularly in studies involving detection of micrometastases and isolated tumour cells (ITCs).[3]

Some molecular methods have been used previously for sentinel node diagnosis, but have shown a lack of reproducibility, a longer time for the intra-operative assessment and an inability to study the whole lymph node.[4] A new molecular method has been developed recently, based on a one-step nucleic acid amplification (OSNA) method.[5]

Our goal was to compare the results of this new OSNA method with the results of conventional histological tests and to evaluate the feasibility of this procedure for the intra-operative evaluation of SN in breast cancer surgery and to evaluate it as a way to help standardize the sentinel node evaluation procedure.

Material and methods

TRIAL 1 (COMPARATIVE HISTOPATHOLOGICAL-MOLECULAR STUDY)

This study focused upon 181 sentinel lymph nodes submitted for intra-operative diagnosis in six different Spanish hospitals. 'Case' refers to the node, not the patient. A maximum of four SN were admitted per patient.

In nine cases, the corresponding tumour was diagnosed as extensive high-grade ductal carcinoma *in situ* (DCIS), while 172 were diagnosed as invasive carcinoma.

All nodes were isolated from the surrounding fat, weighed and measured. The lymph nodes were sliced into parallel 2-mm-thick portions, which were then processed alternately for histological analysis: 'a' and 'c'; 'b' and 'd', following the OSNA procedure.[5]

The histopathological study protocol consisted of touch imprint or H&E staining on frozen sections from each of the sections obtained. The rest of the tissue was embedded in paraffin wax and between one and six pairs of 4-μm-thick sections every 150 μm were prepared according to the protocol of each hospital, one slide stained with H&E and the other with immunohistochemistry (IHC) for AE1/AE3 (Dako®; Glostrup, Denmark).

The OSNA protocol consisted of homogenization of tissue in a mRNA-stabilizing solution (Lynorhag, pH 3.5; Sysmex®, Barcelona, Spain) and subsequent isothermal (65°C) amplification of cytokeratin 19 (CK19) using the Lynoamp amplification kit (Sysmex®) through a reverse transcriptase–loop-mediated isothermal amplification assay (RT–LAMP) in a gene amplification detector RD-100i (Sysmex®) in compliance with the protocol described above.[5,6] The technique uses six primers, which increase the specificity and speed of the reaction.[6] Tissue homogenates from each lymph node were kept frozen at −80°C as a back-up for possible future studies.

All cases were classified according to the tumour–node–metastasis (*TNM Classification of Malignant Tumors*, 6th ed) staging system.[7]

In the OSNA assay, cases showing mRNA CK19 levels >250 copies/μl were considered positive and were classified as micro-metastases (number of copies >250 copies/μl <5000 copies/μl) or macro-metastases (number of copies >5000 copies/μl) following system specifications based on previous calculations.[5] Cases identified as 'negative' (<250 copies/μl) by the system were classified further as ITCs (number of copies/μl >100 but fewer than 250) or true negative if the number of copies/μl was <100.

The results were entered into an electronic database with the following variables: personal data of each patient, number of isolated nodes, weight of each node, number of H&E and cytokeratin (CK) sections, histological diagnosis, OSNA diagnosis, amplification time (reaction rise-time) and number of copies/μl of mRNA CK19 detected by the OSNA assay. The diagnoses obtained by both procedures were then compared via a concordance study between both methods, and the cases in which the choice of treatment (axillary lymphadenectomy or not) would have varied according to the protocol used were analysed further. Three levels of concordance were defined:

1 Level I: 'qualitative and quantitative concordance' in cases where the same diagnosis and the same TNM staging was obtained for both procedures.

2 Level II: 'qualitative but not quantitative concordance' in cases where the same diagnosis was achieved in terms of presence/absence of tumour cells, but which differed in pN staging.

3 Level III: 'discordant' for cases in which diagnosis differed qualitatively.

For cases that remained discordant after review, we performed further testing using the back-up homogenized sample. Western blot and polymerase chain reaction (PCR) analysis of CK19 following the protocol described previously was completed[5] and the remain-

ing tissue was step-sectioned entirely and reviewed after IHC staining for AE1/AE3.

TRIAL 2 (FEASIBILITY OF THE MOLECULAR METHOD FOR INTRA-OPERATIVE DIAGNOSIS)

In this study, we included 55 cases from one of the hospitals participating in trial 1. The entire node was submitted to the OSNA assay in all cases, except in nine cases, which were also included in trial 1, and therefore were studied by both methods. For each of the nodes received, the following data were recorded in an electronic database: the time of extraction in the operating room, number of nodes, time of arrival at the pathology centre, time of node dissection, time of homogenizing and pipetting of sample, time when the sample entered the RD-100i amplification device and time of reporting the intra-operative diagnosis.

Results

TRIAL 1

Table 1 outlines the first review of diagnostic results prior to the study of discordant cases. Level I concordance was observed in 168 cases (92.8%). Of these, 138 were negative for metastasis (76.2%) and 30 were positive (16.5%). Of these, 25 were macro-metastases (13.8%) and five micro-metastases (2.7%).

Three cases (1.6%) showed level II concordance. Two were macro-metastases in OSNA and micro-metastases in histology. One case corresponded to the first of four SNs of a patient who showed 13 000 copies/μl of mRNA-CK19, and the three remaining nodes were negative by both methods. Although this is a macro-metastasis as defined by OSNA, it is located in the lower range of the number of copies, which may correspond to the histological diagnosis of micro-metastasis because of sampling bias. The second case showed positive results in the last two CK sections of tissue adjacent to the section included for OSNA study, also indicating sampling bias. The third case showed micro-metastasis in OSNA and macro-metastasis in the histological deferred trial. There was no record of intra-operative diagnosis in the database, although there was a record of a 3.7-mm metastasis in successive paraffin-embedded sections. The small diameter of metastasis justifies the diagnostic discrepancy, attributable to sampling bias.

Ten cases (5.5%) showed level III concordance (discordant) in the preliminary results (Table 2). Four cases were negative OSNA/positive histology (one macro-metastasis and two micro-metastases). The histological macro-metastases could be demonstrated only in the intra-operative biopsy section and the first level of H&E, not appearing in successive sections. Although no record of diameter could be obtained it was probably a small-volume metastasis, as it appeared in only two successive sections and not in the remaining sections, so we attribute the discrepancy to sampling bias. Two cases showed negative OSNA/micro-metastasis histology. In the first, the metastasis reached a diameter of 0.24 mm, i.e. at the lower limit of micro-metastasis. In the other negative OSNA/micro-metastasis histology case, the OSNA reported isolated tumour cells (number of copies >100 and <250 copies/μl). In both cases the discrepancy was therefore interpreted as due to sampling bias.

Six other 'discordant' cases in the first analysis were positive OSNA (four micro-metastases and two macro-metastases)/negative histology (Table 2). Of the four micro-metastasis OSNA/negative histology cases, three showed a copy number between 250 and 2600/μl, compatible with sampling bias. The fourth case was of a node that was split into three for the OSNA study

Table 1. First review of diagnostic results prior to study of discordant cases

Comparative results	Histological study			
	Macro	Micro	Negative	Total
OSNA				
Macro	25	2	2	29
Micro	1	5	4	10
Negative	2	2	138	142
Total	28	9	144	181

OSNA, One-step nucleic acid amplification.

Table 2. Level III concordance (discordant) in the preliminary results

Level III concordance results (discordant)	Histological study			
	Macro	Micro	Negative	Total
OSNA				
Macro			2	2
Micro			4	4
Negative	2	2		4
Total	2	2	6	10

OSNA, One-step nucleic acid amplification.

Table 3. Results after preliminary analysis

Results after discrepant case analysis	Histological study			
	Macro	Micro	Negative	Total
OSNA				
Macro	32	0	1	33
Micro	0	10	0	10
Negative	0	0	138	138
Total	32	10	139	181

OSNA, One-step nucleic acid amplification.

Table 4. Concordance after analysis of results

Concordance	99.45%
Would not alter decision	0.00%
Would alter decision	0.55%

following protocol (total weight of the node was 1100 mg); one of the three fragments was macro-metastasis using both OSNA and histology (level I concordance); the second showed micro OSNA/negative histology (level III concordance) and the third was negative using both procedures (level I concordance). This case reveals clearly the existence of sampling bias, and therefore we believe that it should be moved from level III concordance to level I.

Of the two macro-metastasis OSNA/negative histology cases, one corresponded to a benign cystic epithelial inclusion and presence of proteinaceous content. The other case was a macro-metastasis by OSNA (13 000 copies/μl), placing it in the lower range of macro-metastasis by number of copies, and this discrepancy may also be due to sampling bias. Results after the preliminary analysis are shown in Tables 3 and 4.

All OSNA-positive cases were also tested using the back-up samples for Western blot and PCR analysis. All cases except one (which will be discussed later) showed concordance with PCR analysis.

TRIAL 2

The overall time spent from receipt of the node(s) until release of the intra-operative report ranged from 26 to 70 min (mean of 39.6 min). In the first phase, 14 nodes were studied and the time elapsed from removal of the SN(s) to intra-operative report ranged from 39 to 70 min (mean 48.5 min) (Figure 1). Once areas for improvement were identified, a specific workflow from the operating room to the Department of Pathology was developed.

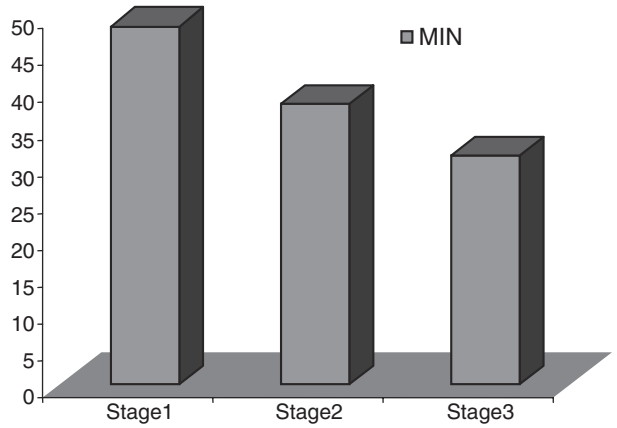

Figure 1. Mean times in the three stages of the learning period.

The workflow starts with the calibration of the system and prior preparation of reagents and test tubes needed. If the pathology department is notified when SN(s) are extracted it is then possible to thaw reagents so that the pathologist and related technicians can begin dissection work immediately upon arrival of the SN(s). Twelve SNs were included in the trial at this stage where the timing reported was a minimum of 36 min and a maximum of 40 min (mean 37.9 min).

The accumulated experience with this workflow led to the progressive reduction of times, which varied eventually from 26 to 35 min (mean 31 min) in the third stage of the trial, which included 29 SNs (Figure 1).

Discussion

After different validation studies, and despite the technical challenges,[1,8] the SN is accepted as the chosen procedure for breast cancer staging in the majority of institutions. The diagnosis should be intra-operative whenever possible to avoid delayed axillary surgery.[2,9] An exhaustive lymph node evaluation and the use of IHC staining improve sensitivity significantly, reducing the false negative rates to approximately 5%.

The prognostic significance of small-volume metastases is contested, although data exist to indicate that SNs with ITCs or metastases identified by IHC may show metastases in lymphadenectomy.[10,11] An exhaustive search of these deposits may be too laborious and expensive to become established routine in a typical hospital centre.

Recently, several studies have focused upon the use of molecular techniques for intra-operative evaluation of the sentinel node. One of these, the GeneSearch trial for breast lymph nodes, uses CK19 together with mammaglobin as targets and has reported a 92.3% concordance rate.[4]

We used a new molecular method (OSNA) to evaluate and compare results in 181 cases from six Spanish hospitals. We found a concordance rate of 99.45%. Other authors, using the same method, have found a concordance rate in the order of 93%.[12–14] Because different tissue slices of the lymph node are compared (alternating tissue portions were included for histology and molecular tests), it is accepted that some tumour deposits, particularly small ones, may be present in one slice and not the next, resulting in discordant results due to sampling bias.[12]

Seven of the ten initially discordant cases in our study, after being analysed carefully by PCR and Western blot using the back-up tissue homogenates, were attributed to sampling bias. When these cases were excluded from the discordant group, two cases still required separate consideration. One of them was diagnosed as a macro-metastasis by the OSNA assay but was negative histologically. In compliance with the protocol, a Western blot of the remaining node homogenates was carried out, which detected the presence of CK19 protein. As it was a small-volume macro-metastasis (13 000 copies/μl), we interpret that the diagnostic difference is probably attributable to sampling bias. The other case was a benign epithelial inclusion, which is an infrequent finding with a positive range for CK19 below the system's cut-off point (<250 copies/μl). We consider this an exceptional case, both for its diagnosis and the morphological characteristics of its inclusion.

In line with the OSNA studies reported previously,[12–14] our sensitivity and specificity results were 98.2% and 94.8%, respectively. Data in the literature support these findings and indicate similar sensitivity and specificity levels between molecular and deferred histological study techniques.[2,4,12] However, each procedure has its advantages and disadvantages.[15] On one hand, deferred histological assessment with immunohistochemistry allows morphological observation of lesions, the evaluation of the location in the node and its two-dimensional measurement, the basis of pN staging. On the other hand, this approach involves the need for a second intervention in cases of metastatic disease. Intra-operative histology studies including immunohistochemistry provide only slightly lower sensitivity and specificity rates, with the added advantage of allowing concurrent axillary lymphadenectomy if needed. However, there is also loss of tissue in the cryostat, with subsequent risk of tumour cell loss.[2]

The experience in Spain is that the histological protocol necessary to achieve good sensitivity rates is difficult to implement given the workload involved. Thus histological study is limited, in most cases, to some H&E tissue sections without immunohistochemical staining, implying that the majority of tissue is left within the paraffin block. In practice, the re-operation rate varied around 20% in the various hospitals (personal communication).

One of the biggest advantages of the OSNA assay versus histological methods is the fact that intra-operative analyses of the whole SN can be performed, allowing immediate decision-making about axillary lymphadenectomy and allowing for the conservation of homogenized tissue material.

Some potential disadvantages of the OSNA procedure are as follows:

1 It does not provide information regarding the location of metastasis within the node, although this is considered an issue for staging only in the guidelines of some working groups.

2 It does not allow morphological evaluation of lesions, but the sensitivity of the procedure and its specificity is a much greater advantage, allowing the detection of ITCs (considered N0 in TNM classification), but of yet uncertain prognostic value. However, our study includes a cystic benign epithelial inclusion (OSNA false-positive) that could have been diagnosed histologically. This entity is extremely rare and generally small and most of them would not reach the positive level established by the system. Our case is exceptional, due to the rarity of diagnosis, the size of its lesion and its cystic morphology.[16,17]

3 Some studies have described mRNA CK19 expression in lymph nodes from patients without neoplastic disease, producing false positives.[18,19] To prevent this outcome, the OSNA procedure establishes a cut-off point above 250 copies/μl, resulting in any illegitimate expression of mRNA CK19 being considered negative by the system, as it falls below the established cut-off.[5,20] Certain haematological disorders could be other possible causes of false positives by molecular biology techniques. CK19 expression can be induced in peripheral blood by cytokines and growth factors that circulate at higher concentrations in inflammation conditions.[18] As a result, false positives are theoretically more likely under these circumstances. However, the positive cut-off point set in the OSNA assay does not take this possibility into consideration.

4 Another possible cause of false positives is the presence of pseudo-genes. At least two pseudo-genes for CK19, namely CK19a and CK19b have been identified, which have significant sequence homology with mRNA CK19.[21] Given that the RT–LAMP includes six different primers and their special design, the specificity of OSNA is 100%.
5 It does not detect CK19-negative tumour cells, possible in cancers whose cells do not express this protein. It has been established[22] that 98.2% of breast carcinomas express CK19. In order to prevent the possible 1–2% false-negative cases due to this circumstance, we recommend the inclusion of CK19 in the immunohistochemistry battery applied to the core biopsy prior to intervention. This would allow identification of possible false-negative cases, which should be excluded from the intra-operative OSNA assay.
6 It does not allow the application of certain histological parameters, such as the Miller and Payne[23] method, for assessing response to neoadjuvant treatment. We believe that until general guidelines are established by general consensus, each Breast Cancer Committee at each hospital must establish its own protocol. In our case, we raised this issue at one of the departments participating in the study. As a result, the Breast Cancer Committee, pending final results on the prognostic value of ITCs, gave more importance to the detection of neoplastic cells than the evaluation of post-neoadjuvant morphological parameters in the Miller and Payne system. This, however, requires further evaluation to establish specific criteria.

Our study also included a timing component designed to determine the step-by-step and comprehensive time breakdown for 55 cases where the OSNA method was utilized. The intra-operative time was found to be dependent upon the number of nodes evaluated and the weight of the nodes being analysed. A defined learning curve was observed, with the longest times monitored for the first 14 cases, regardless of the number of lymph node fragments performed for each case. It was noted that the longest and most variable time-period corresponded to the stage in which the node was transported from the operating room to the pathology department. Upon arrival of the node at pathology, the time of macroscopic processing of the sample(s) could also fluctuate significantly depending upon the training level of the pathologist involved. The least variable time-period corresponded to the homogenization of tissue, preparation of the diluted sample and amplification in the amplification equipment. The reduction in time from extraction of the nodes(s) in the operating room until the generation of the pathology report was achieved primarily by the accurate definition of the workflow and training that was tailored for each of the professionals involved, including the assistants transporting the sample. The consensus and implementation of the new defined workflow established in the second stage of the timing study allowed a gradual reduction of procedure times by more than 10 min on average from the average times recorded in the first stage (Figure 1).

We believe that key factors in reducing time were the consensus among the departments involved in the procedure and the training of particularly motivated staff in the SN diagnosis area. The work protocol is manageable, reproducible and applicable in pathology departments after a period of education and training, including those departments without prior specialist training in the area of molecular biology.

Conclusion

In this study, we have shown that the OSNA is a highly sensitive, specific and reproducible diagnostic technique that may be used for SN pathological diagnostic standardization. We believe that the OSNA method is a new alternative to SN diagnosis, allowing study of the node in its entirety with a high degree of sensitivity and specificity as well as a turn-around time within acceptable limits for intra-operative application.

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