REVIEW

Intraoperative assessment of sentinel lymph nodes in early-stage breast cancer

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Abstract

The assessment of axillary lymph node (ALN) status provides heavily weighing prognostic indicators in deciding on breast carcinoma treatment. In the 6th and 7th editions of the *American Joint Committee on Cancer* (AJCC) *Cancer Staging Manual* are evaluated the nodal metastases based on size and taking into account the number of metastatic cells. According to these *Manuals*, a positive node is equated to metastasis whose size reaches at least 0.2 mm or amounting to more than 200 tumor cells. The clinical significance and the therapeutic optimum of the presence of a minimal nodal involvement after axillary sentinel lymph nodes (SLNs) biopsy remain controversial. The need for further axillary treatment (ALN dissection or axillary radiation) in clinical N0 patients with early-stage breast carcinoma and SLNs metastases remains unclear. In all likelihood, the delivery of the regular adjuvant treatment in association with systemic treatment and radiation therapy results in survival rates similar to axillary treatment completion. This review also presents several assessment methods related to the SLNs at the surgical stage, such as cytological, histological, immunohistochemical and molecular diagnostic techniques, evaluating the advantages and disadvantages of each of them. More studies including larger groups of breast patients are needed to confirm which of them is the most reliable method for the evaluation of the SLNs.

Keywords: sentinel lymph nodes, micrometastases, breast cancer, immunohistochemistry, molecular diagnostic techniques.

⊟ Introduction

It is well recognized that a complete and correct cure of breast cancer involves accurate surgical treatment of both the primary tumor and adequate axillary lymph node dissection (ALND). Complete axillary dissection is often associated with increase morbidity including lymphedema, loss of shoulder mobility and sensory neuropathy.

In an attempt to achieve minimal morbidity in ALND, the intraoperative assessment of the sentinel lymph nodes (SLNs) biopsy is now performed for T1/T2 breast cancer with clinically negative axillae, in many surgical units.

The performance of SLNs assessment aims primarily at the identification of all macrometastases (more than 2 mm) and micrometastases (between 0.2 mm and 2 mm) [1].

ALND can be excluded in the case of clinically node-negative patients showing negative SLNs or in the selected population with limited involvement of the SLNs.

There are several assessment methods related to the SLNs at the surgical stage, such as cytological, histological, immunohistochemical (IHC) and molecular diagnostic techniques.

The frozen section and the imprint cytology represent the most widespread histological intraoperative methods, nevertheless, it has been determined that their sensitivities range from 59% to 91%, and from 30% to 96%, respectively [2].

Due to inconsistent and lower sensitivity, many hospitals do not use intraoperative histological tests.

Some authors demonstrated that the use of immuno-cytochemical procedures could improve the detection of micrometastases on imprint slides [3–6].

Using the rapid IHC procedure, IHC analyses were completed within 16 minutes and the assessment of cyto-keratin (CK)-positive lymph nodes was performed by two pathologists within 20 minutes, the sensitivity, specificity and accuracy being compared with standard IHC [4].

Molecular techniques have the potential to eliminate sampling errors, being more sensitive than frozen section and imprint cytology [2]. Other advantages include the evaluation of a higher volume of the lymph nodes, greater automation, rapidity of tests, cost-effectiveness and non-subjective molecular diagnostics [2].

The main disadvantage is that the histopathological (HP) markers would remain unrecognized [2].

Axillary lymph node (ALN) status becomes a major prognostic factor in patients with early-stage breast cancer, and the assessment of lymph node status is of paramount importance when opting out to deliver adjuvant systemic therapy.

⊟ ALN staging

ALN status is an important prognostic factor being determinant in treatment decision for patients with breast carcinoma. The *American Joint Committee on Cancer*

(AJCC) and the *Union against Cancer* (UICC) were unified in 1987 into a single tumor–lymph nodes–metastasis (TNM) staging system classifying nodal metastases based on size. The 6th edition of the *AJCC Cancer Staging Manual* incorporates extensive and important revisions in comparison to the previous one.

The most visible differences refer to the lymph nodes size, number, location and detection procedures [5, 7].

The *National Surgical Adjuvant Breast and Bowel Project* (NSABP) B32 randomized prospective clinical trial established SLNs biopsy as a risk avoiding and effective method for axilla staging and demonstrates that SLN biopsy plays the same role as ALND in loco-regional control and survival, without morbidity, in patients with T1 or T2 cN0 invasive breast carcinoma [1, 8]. One of the most changes in the 6th edition of the *AJCC Cancer Staging Manual* was to define the limits for micro-metastases as being greater than 0.2 mm but not greater than 2 mm in largest dimension and isolated tumor cells (ITCs), which were described as single cells or small cluster of cells reaching maximum 0.2 mm. A macro-metastasis was classified as one or more tumor deposits greater than 2 mm [7, 9].

In the *AJCC* measurement system of SLNs, tumor deposits were assessed using a micrometer.

When Hematoxylin–Eosin (HE)-stained slides show the absence of metastatic carcinoma, yet, IHC stainings detect ITCs, the classification become pN0(i+) for positive IHC. The labeling of pN1mi(i+) features negative HE staining, but with IHC staining detecting micrometastases, "i" standing for IHC staining [7, 9]. The main difference between the 7th edition of the *AJCC Cancer Staging Manual* and the previous one in regards to SLNs is the count of metastatic cells.

Therefore, ITCs represent single cells or cell clusters of less than 0.2 mm in size and grouping fewer than 200 carcinoma cells in one lymph node section [pN0(i+)], irrespective of the detection procedure. Micrometastases range in size from less or equal to 2 mm to greater than 0.2 mm or aggregate more than 200 carcinoma cells in a single lymph node section (pN1mi), regardless of detection method [10, 11].

In the 7th edition of the *AJCC Cancer Staging Manual*, a positive lymph node is characterized as a metastasis of at least 0.2 mm or counting more than 200 tumor cells [12]. Research has shown that the use of IHC staining on HE-negative lymph nodes is likely to identify micro-metastatic carcinoma in 12–29% of the cases [13–16]. In these situations, the question arises whether to perform ALND completion or not. Kim *et al.* revealed that 48.3% of the SLN-positive patients by HE staining displayed additional nodal metastasis in ALND [17]. In our opinion, complete ALND is recommended in the case of SLN with pN1mi and macrometastasis, but it should be excluded for pN0(i+).

Detection of ITCs was performed in order to prevent overtreatment of low volume nodal involvement; as it is known in some cases, ITCs may be generated by the transport of the passive tumor cell to the SLN, secondary to pre-operative core needle biopsy procedures or fine-needle aspiration. Also, the iatrogenic displacement because of breast massage giving rise to benign epithelial cells in the lymph node, having as a result false positive rates, more particularly in papillary lesions [18].

⊟ Therapeutic and prognostic significance of SLNs with micrometastasis in patients with cN0 early-stage breast cancer

SLN biopsy done without ALND is considered the standard care for SLN-negative patients. For cases with SLN micro- and macrometastasis for T1 and T2 tumors, the complete ALND was the standard treatment [6].

SLN biopsy was not recommended in ductal carcinoma *in situ*, in pregnancy, in the case of large or locally advanced inflammatory breast cancer (T3 and T4) or of prior non-oncological breast or axillary surgical treatment [19].

Many studies have indicated that approximately 60% of SLN-positive patients show no residual disease in the axilla, in consequence having no benefit from ALND and risking complications [20–23].

However, SLN biopsy is coupled with a false-negative rate, as stated in mainstream literature (more exactly in 69 papers) on SLN biopsy validated with ALND, confirming an average false-negative rate of 7.3% [17].

Wei *et al.* identified, in a series of 2043 SLN mapping procedures at their institution, a false-negative rate of 3.1% [24].

In an attempt to forecast the additional ALN implication in patients with limited SLN involvement, many authors took into consideration several clinico-pathological parameters and designed nomograms, *i.e.*, numerical predictive tools which calculate the risk of residual disease in the axilla.

The *Memorial Sloan Kettering Cancer Center* (MSKCC) nomogram uses multiple parameters, such as tumor type, tumor size, nuclear grade, lymphovascular invasion, estrogen receptor status, multifocality, method of intraoperative detection of SLN tumor deposits and number of positive and negative SLNs, in order to generate the probability of residual disease in the remaining ALNs [25–30]. Many practitioners using *MSKCC* nomogram chose no ALND for their patients, which resulted in a nomogram score of 10% or less [31].

The risk of positive non-SLN in patients with micro-metastases is more significant than in patients showing negative SLN (with a risk ranging between 7% and 8%), and less significant than in patients with SLN macro-metastases (the risk being between 30% and 50%) [32]. The clinical significance and the therapeutic option of the presence of a minimal nodal involvement after an axillary SLN biopsy remain controversial. These are some studies which stated that SLN micrometastatic disease was associated with worse prognosis.

In their study, Weaver *et al.* (2011), focusing on the prognosis of SLN micrometastases, in patients enrolled to the *NSABP* B32 clinical trial, found a significant difference in five-year overall survival (OS) and in the five-year recurrence-free survival (RFS) between pN0 patients and those with SLNs micrometastases. The difference was even sharper when the size of the lymph node metastasis increased [33].

In 2010, de Boer *et al.* stated that micrometastases of the lymph nodes, as detected by HE staining in one

section of each ALN, were associated with lower values of OS and disease-free survival (DFS) [34].

On the other side, several studies showed no significant differences between the results of patients with minimal axillary nodal involvement and those with no axillary nodal disease [35–39].

The *American College of Surgeons Oncology Group* (ACOSOG) Z10 trial did not find a major difference between patients with micrometastatic disease in axillary lymph nodes and those without axillary nodal involvement [36–39]. In this studies, patients with minimal axillary nodal involvement were administered systemic treatment, mostly due to the association between micrometastases in axillary nodes and unfavorable prognostic factors (such as age, tumor grade or tumor size) [32].

Maaskant-Braat *et al.* (2011), in a Dutch study involving a median follow-up of 50 months, discovered no major difference of survival rates for patients with SLN micrometastatic disease and those without SLN involvement, even following the adjustment of prognostic factors, such as age, tumor size, grade, systemic treatment or not [37].

In conclusion, we can rightly state that the SLNs micrometastases have been associated with an increased recurrence risk and inconsistently connected to poorer survival, but their prognostic significance can change depending on the molecular subtype [32].

The presence of micrometastatic lymph node involvement after a SLN technique raised two questions:

(*i*) What is the risk to leave residual disease in ALN for the patients with SLN biopsy without further axillary treatment?

(*ii*) Is the minimal nodal involvement a sufficient argument to select an adjuvant systemic treatment?

The *International Breast Cancer Study Group* (IBCSG) 23-01 trial stated no significant difference in DFS between patients with T1 or T2cN0 breast carcinoma and SLN micrometastases with and without ALND [10].

The *ACOSOG* Z0011 prospective randomized trial evaluated the benefit of ALND in the case of patients with one or two positive SLNs.

As far as the 6.3 years median follow-up, there were no major differences in regional lymph nodes recurrence, DFS or OS being recorded between patients with ALND and those without ALND [40, 41]. The results of Z0011 study pointed out that patients with T1,T2 tumors with two or fewer positive SLNs, undergoing breast-conserving surgery (BCS) and whole-breast irradiation do not benefit from ALND [41].

The guidelines published by the *American Society of Clinical Oncology* (ASCO) in 2014 favor no completion ALND for patients with fewer than three positive SLNs, unless there is evidence of bulky metastatic disease or gross extracapsular extension (ECE) and the patient undergoes whole-breast irradiation [42].

Vestjens *et al.*, in the *Netherlands Cancer Registry Study*, in 2012, highlighted a higher five-year axillary recurrence (AR) rate in patients with SLN micrometastases who have not undergone ALND, in comparison with those to which ALND was performed (5.6% *vs.* 2.3%, respectively). The authors also stated that the adjuvant treatments, such as systemic treatment and breast irradiation, significantly lowered this risk [12].

The <u>A</u>fter <u>M</u>apping of the <u>A</u>xilla: <u>R</u>adiotherapy <u>O</u>r Surgery? (AMAROS) prospective randomized clinical trial evaluated the management of the axilla in T1,T2, cN0 patients with a positive SLN, these patients being randomized to ALND or axillary radiotherapy. The results showed no significant differences in AR, DFS or OS between the two groups [43]. The patients submitted to ALND displayed a higher incidence of lymphedema at five years compared to patients undergoing regional radiotherapy (23% *vs.* 11%, respectively), but the quality of life was of the two groups involved did not differ to a large extent.

The *AMAROS* study does not state that further axillary treatment is required for all patients with positive SLNs [43]. Metastatic carcinoma can pass through the lymph nodes capsule to the surrounding axillary fibroadipose tissue.

Several studies established that in the case of cN0 patients with early-stage breast carcinoma, the focal ECE occurs in the SLNs of 19% to 30% of them [44–46]. ECE is linked to poor prognosis and is closely correlated with other negative prognostic factors, such as SLN macrometastases and lymphovascular invasion [46–48]. As far as patients showing ECE to an extent larger than 2 mm are concerned, they are more prone to have additional positive nodes than those with ECE 2 mm or less. These data indicated that ECE having an extent larger than 2 mm may require further axillary treatment [1, 46].

The need for further axillary treatment (ALND or axillary radiation) in cN0 patients with early-stage breast carcinoma and SLN micrometastases remains unclear. In all likelihood, the delivery of the regular adjuvant treatment in association with systemic treatment and radiation therapy results in survival rates similar to completion axillary treatment [49].

In the *AMAROS* trial, factors such as the patient age, tumor grade, size of SLN metastasis, macrometastases and multifocality, were interconnected with chemotherapy initiation, which is not the case of the number of positive nodes [50].

From all tumor biological criteria, the tumor grade ranked topmost in establishing the adjuvant systemic treatment, followed by human epidermal growth factor receptor 2 (HER2) status and non-SLNs positivity for low grade, HER2-negative tumors [32].

In conclusion, we can state that the AR registers a low rate (<2%) even in the cases without ALND, despite a non-SLN positivity ranging from 10% to 18%, in association with adjuvant treatments, such as chemotherapy, radiotherapy or hormonal therapy.

⊟ Intraoperative assessment of SLNs in breast cancer

The current assessment methods in relation to the SLN during surgical treatment involve cytological and histological techniques [51]. There is a variation in local histological practice among different surgical centers. The histological frozen section is the most commonly used technique but there are some Units employing a combination of imprint cytology and frozen section or imprint cytology alone [51].

In published literature, the specificity of frozen section consistently approached 100%, indicating that the false-

positive rate with frozen section is close to zero, but the reported sensitivity ranges from 57% to 74% [23, 52–54]. Frozen section is expensive, labor intensive, requiring a histopathologist for each surgical session.

Intraoperative imprint and scrape cytology, belonging to cytological techniques, outrate frozen section analysis. The main advantages lie in the shorter preparation time, lower cost of cytological specimens and no loss of tissue when compared to the frozen section [50]. The estimated specificity for imprint cytology is 99% but the sensitivity is less than for frozen section, ranging from 33% to 73% [55–57]. False negative cases of imprint cytology occur more frequently in invasive lobular carcinoma and in the presence of micrometastatic disease [51, 55].

The benefit associated with in frozen section sensitivity could be dealt with by using a larger number of slides for imprint cytology.

Some authors demonstrated that the use of immuno-cytochemical procedures could improve the detection of micrometastases on imprint slides [3]. However, immuno-staining is less practical for intraoperative use, has a not clearly defined role in the intraoperative staging of SLN biopsy, being time consuming and expensive, the standard IHC protocols requires 2–4 hours to complete [4].

Terata et al. designed a state-of-the-art device enhancing rapid IHC (R-IHC) analyses in approximately 20 minutes, based on alternating current (AC) electric field [4]. Using this procedure, IHC analyses were carried out in less than 16 minutes, while the examination of CK-positive lymph nodes by two pathologists lasted about 20 minutes [4]. The AC electric field accelerated the antigen–antibody reaction [58].

What are the most important advantages of intra-operative R-IHC method?

First of all, R-IHC has better sensitivity, specificity and accuracy than conventional HE staining alone, being compared to standard IHC (95.2%, 100% and 99.4% for IHC procedure, while for intraoperative HE staining were 76.2%, 100% and 96.9%, respectively) [4]. Another important mention concerns cost effectiveness, R-IHC enabling the same accurate diagnosis as when using main molecular techniques, such as one-step nucleic acid amplification (OSNA), but the cost is less than a quarter of OSNA. The high cost of IHC analysis is due to the primary antibodies are expensive but Toda et al. stated that through R-IHC the concentration of primary antibody could decrease by more than 90% [58].

When examining HE-stained sections under microscope, it is possible that pathologists may not detect metastatic lesions. On the contrary, they can readily detect metastatic lesions when using R-IHC, even when low-power field is involved (e.g., 40×). This technique optimizes the time required for intraoperative diagnosis, as well as the effort to detect unclear lesions like micrometastases, ITCs and artifacts of frozen section [4, 59]. R-IHC could be useful for SLN biopsy after neoadjuvant chemotherapy, this therapy being now used increasingly for early-stage breast cancer [60].

NSABP B18 clinical trial revealed that the detection of micrometastases following neoadjuvant chemotherapy counts as a poor prognostic factor [61].

By applying R-IHC to intraoperative SLN biopsy in patients with breast cancer, the specimen is preserved, enabling additional examinations, such as the accurate identification of the subtype of lymphatic metastases, the presence of extranodal spread or any other additional HP or molecular investigation [4].

The diagnostic workups using morphopathological changes can be also substituted by molecular diagnostic techniques.

These techniques of proteomics, genomics and meta-bonomics could be considered viable solution replacing histomorphological tests [62, 63]. Standard histological procedures focus only a small part of the total volume of the SLNs, running the risk of serious sampling error due to non-examining the part of the lymph node containing metastasis.

Molecular techniques are likely to avoid sampling errors. The main techniques of genomics, such as quan-titative reverse transcription–polymerase chain reaction (qRT-PCR) and OSNA, are based on the identification of messenger ribonucleic acid (mRNA) for marker genes, which are overexpressed in tumor cells but are not visible in the normal tissue [51].

The potential of these two techniques of reducing or eliminating sampling error depends on the size of the tissue referred to HP examination.

Taking into consideration that qRT-PCR underpins homogenization of the sample tissue, the HP examination of the tissue and direct comparison are excluded [51]. The studies suggest that these molecular assays prove to be more sensitive than frozen section and imprint cytology, with regard to the intraoperative SLNs examination [2].

Most likely, this kind of molecular techniques will supersede HP examination as a standard method for detection of metastasis.

The main benefits relate to the examination of a larger volume of the lymph node, higher automation, enhanced speed of the tests, cost effectiveness and objectivity of molecular diagnostics. However, resorting solely to molecular techniques, loss of HP markers, such as the extent of metastatic deposits and of extranodal or extra-capsular spread, which, as a rule, guide the oncological practice, would remain undetected.

Furthermore, the HP features of the primary metastasis index can be used exclusively to distinguish between recurrence and a new focus of primary disease. The high-resolution magic angle spinning (HRMAS) proton magnetic resonance spectroscopy (MRS) has proved to be a reliable metabonomics technique, and it furthers the examination of the metabolic profile of an intact tissue [64]. This method is relied on to discriminate between malignant breast tissue and adjacent normal tissue, being a state-of-the-art efficient method of tumor identification in the SLN biopsy specimen with early-stage breast cancer patients. The in vitro use of HRMAS on intact lymph node slice within 30 minutes from operating can successfully replace the frozen section HP examination [65].

Choline and choline-containing compounds, such as phosphocholine and phosphatidylcholine, are encountered in several tumor types, yet, these metabolites are not present in normal and benign tissues. Choline and its derivates can be said to lay the foundations of cell physiology and to accelerate cell proliferation in the case of malignancy [65–67]. Choline and choline-containing compounds signal is considered to be less important in

patients with breast cancer undergoing neoadjuvant chemotherapy [68].

Another important metabolite detected in breast cancer tissue is lactate. In the case of malignant cells, the anaerobic metabolism of glucose represents the major metabolic process and is liable for the increased level of lactate [65]. The joint presence of choline, lactate and other metabolites, such as taurine, *myo*-inositol succinate, β-glucose and glycine, were considered as a sum total marker of malignancy with greater diagnostic sensitivity in tissue samples [66].

The tissues specimens used for HRMAS analysis can be formalin-fixed, paraffin-embedded and sent for HP examination. Therefore, the metabolic profiles of the nodes can be associated with the regular HP outcomes.

The studies comparing HRMAS results with classical HP test using HE staining showed a very high correlation, conferring to this molecular technique a higher sensitivity of detecting malignant tissue within dissecting lymph nodes than frozen section HP examination.

In a study carried out in 1997 on breast cancer biopsies from 191 patients, Mackinnon *et al.* stated that the MRS could be exploited to differentiate between benign and malignant tumors, showing a high degree of sensitivity (95%) and specificity (96%) [69].

Several other applications of *in vitro* HRMAS studies include the accurate identification of residuals both in the tumor bed and margins, detection of metabolites with reference to tumor aspirate, brush cytology specimens, fluids such as nipple discharge, ascitic tap or detection of micrometastases [66]. The major strengths of this state-of-the-art technique appear to be fast and easy sample handling, the total analysis time being between 15–30 minutes, which is highly competitive with frozen section HP examination.

On the other side, MRS equipments are generally expensive and are not yet largely available in the most of public hospitals, being now available in the pharmacological and chemical industry, which commonly employ MRS to assess the purity of their products. Considering how widespread surgical interventions for breast cancer are, there would be a need for significant number of Units able to perform HRMAS to cater to all the breast operating centers in order that the HRMAS facility to be promoted as a cost effective technology. More studies including larger groups of breast patients are needed to confirm the value of HRMAS as a reliable method for the evaluation of the SLNs.

⊟ Conclusions

The ALN status is a critical predictive factor in the treatment decision-making concerning breast carcinoma patients. The clinical significance and the therapeutic option of the presence of a minimal nodal involvement after an axillary SLN biopsy remain controversial. The AR rate has been proved to be low in patients with SLN micrometastases, these cases being inconstantly related to a poor survival, but their prognostic significance can be different according to the molecular subtype and in relation to adjuvant treatments. More studies including larger groups of breast cancer patients are needed for evaluating the advantages and the disadvantages of several methods for the SLNs intraoperative assessment.

Conflict of interests

The authors declare that they have no conflict of interests.

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