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REVIEW ARTICLE

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Pathologic examination of the axillary sentinel lymph nodes in patients with early-stage breast carcinoma: current and resolving controversies on the basis of the European Institute of Oncology experience

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Abstract Several controversial aspects of sentinel lymph node biopsy (SLNB) for patients with early-stage, node-negative breast carcinoma have been dealt with and resolved in the past decade since its introduction. Unfortunately, however, there is still no consensus on how best to examine sentinel lymph nodes (SLN) histologically. As a consequence, the protocols for SLN examination are remarkably variable in different institutions, leading to a very poor reproducibility of the data stemming from investigations on series of patients whose SLNs have been evaluated according to diverse protocols. Patient outcomes, however, can be optimised only by standardization of the whole procedure of SLNB, with particular reference to the histopathologic scrutiny. Lack of a standardized histopathologic protocol likely derives also from the uncertainties about the clinical implications of minimal lymph node involvement (isolated tumour cells and micrometastases) with regard both to the risk of additional metastases to non-sentinel lymph nodes of the same basin and to the prognostic value for patients' survival. This review aims at highlighting some of the controversial issues of the histopathologic examination of the SLNs, including the number of sections and cutting intervals, the use of immunohistochemistry and the role of molecular biology assays.

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Introduction

Over the past decade, lymphatic mapping and axillary sentinel lymph node biopsy (SLNB) have been increasingly adopted for staging with minimal morbidity patients with node-negative, early-stage breast carcinoma. Since the procedure was introduced, several problem aspects and controversial issues have been dealt with and resolved, while others still remain open. The studies reported thus far support the validity and safety of SLNB in different clinical scenarios where they were formerly questioned, as for patients with previous surgical biopsy/lumpectomy or with multicentric breast carcinomas [39] and for patients treated with neoadjuvant chemotherapy [27]. The safety of the procedure in pregnant women and the accuracy of a second SLNB for relapsing tumours following conservative surgical treatment inclusive of SLNB remain to be assessed.

Several methodological issues regarding the site of tracer injection, the type of tracer, and the surgical identification and removal of the sentinel lymph node (SLN) have also been clarified, and guidelines or procedures for lymphatic mapping and SLN removal have been agreed upon. It is surprising that, despite its pivotal role in determining the accuracy and hence the clinical utility of SLNB, the histopathologic examination of SLN largely remains an unresolved issue.

To be effective in properly staging with minimal morbidity patients with clinically node-negative breast malignancies, the SLNB must rely upon a very accurate histopathologic examination of the SLNs [7, 42, 45]. It is indeed intuitive that the detection rate of metastatic tumour depends on the accuracy and extent of the histopathologic evaluation, i.e. the number of sections scrutinized. The lack of universally adopted protocols for the examination of SLNs resulted in a wide heterogeneity of the procedures currently used in different institutions, and in several guide-

lines or recommendations issued by scientific organizations at the local or international level. Unfortunately, this led to a very poor reproducibility of the data stemming from investigations on different series of patients whose SLNs have been evaluated according to different protocols. As a result, despite almost a decade of investigations on SLNB and several thousand patients enrolled in clinical studies, a number of questions still remain unanswered. Among these, the most important is whether or not the histopathologic examination of SLNs should aim at detecting even minimal lymph node involvement (isolated tumour cells and micrometastases) using all the available technological resources, from the traditional step sectioning with or without immunohistochemical detection of specific tumour cell markers to more sophisticated assays [e.g. reverse-transcription polymerase chain reactions (RT-PCR)]. This question, however, may only be addressed properly after the clinical implications of minimal lymph node involvement are definitively assessed, with regard both to the risk of additional metastases to non-sentinel lymph nodes of the same basin and to the prognostic value of isolated tumour cells (ITC) and micrometastases for patients' survival [18, 38]. Indeed, due to the lack of a standardized histopathologic examination, the clinical implications of minimal SLN involvement are still debated, with some studies documenting an important risk of additional involvement of higher-echelon lymph nodes and of unfavourable clinical outcome, at variance with the results of other investigations [9, 10, 17, 23–25, 30, 32, 33, 35].

Due to the uncertainty on the clinical implications of minimal SLN involvement, individual institutions have reserved the right to devise their own protocol for SLN examination according to the available resources and the cost/benefit analysis. A recent survey of the protocols used in Europe for the histopathologic examination of axillary SLNs of breast carcinoma patients has unveiled remarkable differences among the 240 laboratories that responded to the questionnaire, with the reporting of 123 somewhat different histological protocols. Almost 12% of the responding laboratories examined only one level per SLN, whereas all the other laboratories performed a multilevel assessment, ranging from the evaluation of 2–5 levels to more than 100 levels separated by 40-μm cutting intervals. Patient outcomes, however, can be optimised only by standardization of the whole procedure of SLNB, with particular reference to the histopathologic scrutiny [8, 22].

The histopathologic examination of SLN

The very first histopathologic approaches to SLN examination did not differ from the routine assessment of the regional lymph node status, based on the evaluation of a single or very few sections cut from frozen (in case of intraoperative diagnosis) or paraffin-embedded lymph nodes. As expected, however, the high false-negative rate of this traditional approach was soon highlighted by the first reports on the higher accuracy of a more extensive histopathologic examination of SLNs.

Subsequently, a large series of studies aimed at assessing the benefit of a more extensive histopathologic examination of SLNs. Endpoint of the studies was to increase the positive and negative predictive value of the SLNB by using more demanding histopathologic protocols based on sub-serial sectioning of the entire SLNs and the use of ancillary immunohistochemical staining techniques [21] that however had to be cost-effective and not exceeding the workload capacity of the laboratory. It is self-evident that the cost/benefit analyses of an extensive examination of SLNs vary according to several parameters, including the number of patients evaluated, the amount of the reimbursement and the availability of dedicated personnel and instrumentation. As a rule, the detection rate of metastases depends on the number of sections examined from the entire lymph node, so that the prevalence of SLN involvement in a given series of patients depends on the adopted histopathologic protocol. To ensure optimisation of patients' management in different institutions, however, a standardized protocol should be agreed upon [46, 49].

We have devised one of the most demanding protocols for SLN examination of breast carcinoma patients, which requires the histopathologic scrutiny of the entire SLN by step sectioning at very thin cutting intervals. According to this protocol, which is suitable for both frozen and paraffin-embedded lymph nodes, the SLN is bisected along the longest axis or cut into 2- to 3-mm slices if thicker than 5 mm (thinner lymph nodes are processed uncut). In handling the lymph node, special attention should be paid to preserve intact the nodal capsule and the peripheral sinuses, where many metastatic foci may be first seen. Sections are then cut at 50- to 100-μm intervals until the complete examination of the lymph node by scanning individual sections at 100× magnification. Spare sections for immunohistochemistry were formerly cut at each level, but now we perform immunohistochemical reactions on destained sections whenever deemed necessary to confirm the metastatic nature of morphologically atypical cells (approximately 5% of the cases).

The need for a complete examination of the SLN derives from the observation that while larger (macro-)metastases, measuring 2 mm or more, are detected in the vast majority of the cases in the very first sections cut from the middle areas of the nodes, smaller (micro-)metastasis, measuring less than 2 mm, are more randomly distributed throughout the SLN and cannot be reliably detected by the examination of the sections from the central portions of the node. The choice of cutting step sections at 50- to 100-μm intervals is dictated by the aim of identifying even a minimal lymph node involvement (ITC and micrometastases), which could escape detection by sectioning the node at wider cutting intervals [45, 46].

That such a labour-intensive protocol could not be followed by many other institutions is intuitive, especially when considering that the clinical implications of minimal SLN involvement are still controversial. Thus it is not surprising that recommendations for SLN examination issued by nationwide or international organizations suggest more affordable protocols [8, 22], which re-emphasize the

need for a complete examination of the node, but allow cutting intervals ranging from 0.5 [22] to 1 mm [8]. The rationale for defining these minimal requirements is only to assure the detection of macrometastases and not to search for minimal lymph node involvement by ITC or micrometastases. Accordingly, use of ancillary diagnostic techniques, such as immunohistochemistry or molecular biology assays, is not recommended. Similar conclusions were reached by the panelists of the Consensus Conference held in Philadelphia in 2001 [37]. Whether these recommendations will be maintained or changed depends mainly on the future progresses in unveiling the clinical implications of minimal lymph node involvement.

Workload for the department of pathology

At first glance, any protocol for a more or less extensive examination of SLNs looks very worrisome for the increased workload for both the histo-technicians and the pathologists. In several institutions, the pathologists' reaction to the implementation of the SLNB for breast carcinoma patients is to declare the unavailability of sufficient human and/or instrumental resources for an extensive examination of the SLNs. This might well be true in rare instances, but in the majority of the cases, the SLN workload should be affordable.

The above-described protocol for SLN examination implemented at the European Institute of Oncology requires that approximately 60 sections are cut from each individual 0.5-cm-thick SLN and examined histologically. This might well seem a painstaking procedure, not affordable in many other centres. The first reaction, however, could be mitigated by considering that in about 70% of the cases, the SLN will be free of metastases, thus preventing any further axillary treatment. In these circumstances, the workload for SLN examination will be counterbalanced by the avoidance of embedding, trimming, cutting and examining sections from each of the 20–30 lymph nodes retrieved by complete axillary clearing.

In approximately 60% of the positive SLNB, the size (>2 mm) of the metastases will allow their detection in the very first sections of the SLN (obtained from the central portion of the nodes, after bisection) or even by touch cytology, and the examination of further sections will be spared. Although these patients will undergo completion axillary dissection, the additional workload for SLN examination may indeed be very limited. In the remaining 40% of the cases, the SLN will be minimally involved by micrometastases or isolated tumour cells whose detection requires a complete examination of the SLN by step sectioning. The diagnosis of minimal SLN involvement will be followed—at least in some institutions—by completion axillary dissection, resulting in a net increase in the workload for the laboratory. It should be noted, however, that this applies to only about 12% of the patients with early-stage breast carcinoma.

From our experience with more than 8,000 SLNB examined in the last 9 years, we have calculated that our procedure implies an overall additional workload for the laboratory of approximately ten sections per treated patient (considering that almost 75% of the patients undergo SLNB). A slight modification of the protocol (i.e. cutting step sections of the whole SLN at 100-μm intervals) would be enough to eliminate the need for any additional section. These figures depend on the fact that we routinely scrutinize non-sentinel axillary lymph nodes by examining three to six sections per node. The mean number of SLN per patient is 1.5 (median 1 SLN/patient), the mean number of lymph nodes retrieved by axillary dissection is 27, and the patients with minimal SLN involvement are offered randomisation in a clinical trial comparing axillary dissection with no further treatment. Needless to say, the above conditions vary in different institutions because the mean yield of a completion axillary dissection, the number of sections examined from non-sentinel axillary lymph nodes, the mean number of SLN removed per patients and the mean thickness of the SLNs are highly variable and prevent any general statement to be made. The take-home message, however, is that a truly extensive histopathologic examination of SLNs may well have a lower impact on the global workload of pathology departments than figured and feared at first glance.

False-negative and false-positive SLNB

The assessment of SLN status may be affected by both false-negative and false-positive histopathologic results.

A false-negative SLNB is almost invariably due to a less-than-optimal scrutiny of the SLN, either because of the number of evaluated sections is not high enough to be truly representative of the node status or because metastases escape recognition by the pathologist. The issue of a variable detection rate of metastases according to the more or less extensive examination of the SLNs has been dealt with in the preceding paragraphs. It is quite uncommon for an experienced pathologist to overlook lymph node metastases. There are instances, however, where small and/or morphologically unusual metastases from breast carcinoma may be misinterpreted as non-neoplastic resident cells, most likely nevus cells [34], epithelioid histiocytes of granulomatous lymphadenitis or vessels with high endothelial lining, especially when examining frozen tissue sections. To avoid misdiagnoses, use of ancillary immunohistochemical techniques for specific markers of the epithelial lineage (i.e. cytokeratins) may be extremely useful. These techniques may also be applied to intraoperative frozen section diagnosis, taking advantage of commercially available reagents that allow the reaction to be completed in less than 20 min [5, 41].

Less attention has been paid to the risk of false-positive identification of SLN metastases, although this would imply unwarranted lymph node dissection and improper adjuvant treatments.

Carter et al. [4] first warned of the possible occurrence of passive transport to the axillary lymph node of normal (and neoplastic) breast tissue following diagnostic needle

procedures on the breast. Chiu et al. [6] emphasized inaccurate immunohistochemical procedures as a potential source of false-positive SLNB. Furthermore, the immunoreactivity for low-molecular-weight cytokeratins of fibroblastic reticulum cells invariably present in normal lymph nodes [11, 14] may be responsible for a false identification of metastatic cells. A more subtle cause of false-positive identification of breast metastases is the possible occurrence of benign-appearing breast tissue in the lymph node capsule and/or parenchyma.

The occurrence of ectopic non-neoplastic tissue within lymph nodes is a well-recognized phenomenon, especially for thyroid or salivary structures within latero-cervical lymph nodes or nevus cells in lymph nodes from different basins [13]. Mammary glands are the ectopic epithelial structures most frequently detected within axillary lymph nodes [19], and at variance with most of the aforementioned examples that occur intra-parenchimally, these are commonly seen in capsular or sub-capsular locations or in the peri-nodal fat tissue. Occasionally, the ectopic tissue has been interpreted as metastatic, thus leading to over-staging and, eventually, over-treatment of the patients [13, 19].

We [26] first reported on the occurrence of benign epithelial inclusions of ectopic breast tissue in axillary SLNs of seven breast carcinoma patients, with remarkably different morphological features. Indeed, these benign epithelial inclusions may undergo proliferative changes similar to those encountered in fibrocystic disease (florid duct hyperplasia and sclerosing adenosis), making the differential diagnosis with true metastases very difficult. Again, use of appropriate immunohistochemical reactions for specific markers (e.g. p63, smooth-muscle myosin, calponin) of the myoepithelial cell component, invariably present in benign inclusions and absent in metastatic breast carcinoma, is instrumental in reaching the correct diagnosis, but the examining pathologist should be fully aware of the possible occurrence of such benign inclusions.

Predicting the status of non-sentinel lymph nodes

The extensive examination of SLNs has significantly increased the detection rate of minimal lymph node involvement, and the question has now arisen whether or not micrometastases or ITC only in the SLN predict a risk for additional non-sentinel node metastases high enough to justify completion axillary dissection for these patients. Previous studies had suggested that breast carcinoma patients with minimal SLN involvement could indeed be spared axillary surgery because of an alleged negligible risk of additional metastases, but we and other authors documented a 20–24% risk of involvement of further-echelon lymph nodes in patients with micrometastatic SLNs [40, 49].

More recently, we evaluated a large series of patients with positive SLN biopsy followed by completion axillary dissection, showing that of the 116 patients with ITC only in the SLN, 17 (14.7%) had further axillary involvement, as did 68 (21.4%) of the 318 patients with SLN micrometastases (0.2–2 mm in size) [47]. The prevalence of additional metastases was not significantly different between these two groups of patients (P=0.15), whereas patients with macrometastatic disease in the SLNs showed a significantly higher proportion of non-sentinel lymph node metastases, which were detected in 50.3% (399/794) of the cases (P<0.0001). However, when the patients with SLN micrometastases were further stratified according to the size of the micrometastases (up to 1 vs 1–2 mm), those with larger micrometastases showed a significantly higher prevalence of additional metastases (30.2 vs 17.0; P=0.01), thus confirming previous data [17, 47]. To summarize, patients with a positive SLN biopsy can be stratified in three groups at significantly different risk for involvement of non-sentinel lymph nodes. Patients with ITC or small (up to 1 mm) micrometastases in the SLN have the lowest risk of additional metastases (16.2%), which increases to 30.2 and 50.3% for patients with 1–2 mm micrometastases or larger metastases, respectively.

In each subgroup of patients with SLN metastases, the identified additional metastases to non-sentinel lymph nodes were mostly of the macrometastatic type, i.e. larger than 2 mm [47], most likely because the non-sentinel nodes were scrutinized with a limited number of sections (3–6) and at least some micrometastases or ITCs were likely missed. Therefore, completion axillary dissection in all the patients with positive axillary SLN biopsy may add clinically meaningful information because in the vast majority of the cases, including those with ITC only or micrometastases in the SLNs, non-sentinel nodes will be shown to harbour metastases larger than 2 mm.

To ascertain whether the identification of minimal SLN involvement (with the associated risk of additional non-sentinel lymph node metastases) is per se informative enough to plan an effective adjuvant treatment without the need for further axillary surgery, the International Breast Cancer Study Group has launched a randomised clinical trial (IBCSG trial 23-01), whereby patients with micrometastatic disease or ITC only in the SLN are randomised to further axillary surgery or follow-up. Primary endpoint of the trial is disease-free survival; secondary endpoints are overall survival and quality of life.

Tumour mRNA markers

The ever-increasing adoption of SLNB for staging patients with minimal morbidity has raised the question of how best to evaluate the SLN to minimize the risk of false-negative results. Owing also to the lack of standardized and widely accepted protocols for a truly extensive histopathological examination of SLN, the relative merits of alternative assays based on the identification of tumour-specific mRNA markers over traditional histological or immunohistochemical methods have been extensively exploited. Initially, qualitative RT-PCR experiments documented that several mRNA markers, either alone or in multimarker assays,

were indeed suitable for a high-sensitivity detection of metastatic breast cancer or melanoma in sentinel or non-sentinel lymph nodes [2, 3, 28, 31].

The specificity of the assays, however, was less satisfactory, with a high prevalence of positive assays in histologically unaffected lymph nodes and also in lymph nodes obtained from patients without malignancies [2]. Because a possible cause of false-positive identification of lymph node metastases by RT-PCR assays might be the illegitimate expression of low levels of target mRNAs by non-neoplastic cells, real-time quantitative RT-PCR (qRT-PCR) assays have been developed to correctly identify lymph node metastases according to a threshold level of mRNA expression [15, 20, 29, 36, 48]. Very recently, dedicated kits and instruments for "rapid" quantitative RT-PCR assays have also become commercially available for the intraoperative detection of SLN metastases from breast carcinoma.

A critical reappraisal of the investigations dealing with the identification of breast carcinoma mRNA markers in axillary sentinel and non-sentinel nodes, however, still challenges the possible application of qRT-PCR assays in the clinical practice. Indeed, in the studies with the largest series of patients evaluated, the overall concordance between qRT-PCR assays for individual or multiple mRNA markers and morphological or immunohistochemical findings ranges from 73.2 [15] to 95.7% [48]. Discrepancies include both false-negative qRT-PCR results, with histologically detected metastases escaping identification by the assays and still positive assays in lymph nodes not harbouring any morphologically identifiable metastasis.

Besides the choice of the molecular assay (qualitative vs quantitative RT-PCR), a most likely cause for the discrepancies between morphological and molecular findings is the sampling procedure, with part (or half) of the lymph node being subjected to molecular biology assays and another part to morphological and immunohistochemical examination. As a consequence, the smallest tumour deposits (micrometastases and ITC) may remain confined exclusively to the sample undergoing one assay, escaping detection by the other.

To minimize the effects of sampling on the final results, we have combined RT-PCR assays with our procedure whereby axillary SLNs of patients with breast carcinoma are snap frozen and examined completely by step sectioning at 50-μm intervals. While the frozen sections are examined histologically (including immunohistochemical testing), the interval tissue is subjected to RT-PCR assays. Applying this procedure, we have previously examined 146 axillary SLNs from 123 patients for the detection of five different mRNA tumour markers (cytokeratin 19, CEA, mammaglobin 1, MUC-1 and maspin) by qualitative RT-PCR assays [28]. When analysed individually, the general concordance with the histopathologic findings ranged from 78.8 to 83.6%, with none of the different markers attaining a sensitivity higher than 77.8%. Mammaglobin (MGB1) was the mRNA marker most closely correlated with the histopathologic findings, with a sensitivity of 77.8% and a specificity of 86.1%. We have more recently evaluated the effectiveness of a real-time qRT-PCR assay for MGB1 mRNA in the detection of lymph node metastases in a retrospective series of 81 axillary SLNs from 72 patients, for which the results of the previous qualitative assays were known, and validated the results in a prospective series of 61 SLNs from 61 newly diagnosed breast carcinoma patients.

In our experimental conditions, qRT-PCR assays were indeed more accurate than conventional RT-PCR in assessing the status of axillary SLNs: four of the six false-negative cases in conventional RT-PCR assays turned positive in qRT-PCR experiments, whereas two of the five false-positive cases in conventional RT-PCR assays turned negative in qRT-PCR experiments. Accordingly, qRT-PCR assays were superior to qualitative RT-PCR in terms both of specificity and of sensitivity.

The results were compared with the histopathological findings obtained by a very extensive examination of the same lymph nodes, specifically designed to minimize the possible effects of the sampling procedures on the correlation between morphological and molecular data. The overall concordance with histopathological findings was 93.8 and 93.4% in the retrospective and validation series, respectively, well in line with previous findings from qRT-PCR assays of individual or multiple tumour markers, indicating a concordance ranging from 73.2 [15] to 95.7% [48]. The overall sensitivity of the assay in the two combined series was 88.5% and the specificity 94.8%, with a PPV of 79.3% and an NPV of 97.3%. The false-negative rate of the assay (11.6% or 3/26 histologically positive SLN), and the rate of positive detection of MGB1 mRNA (5.2% or 6/116) in lymph nodes not harbouring any histologically identifiable metastasis, however, question its use in the clinical practice as an alternative to the histopathological examination of the SLN. In the above study, we compared the qRT-PCR findings with a very extensive histopathological evaluation of the SLN, which makes it unlikely that the molecular assay actually identifies true metastases missed at the morphological scrutiny. Whether the molecular assay may be a valuable and cost-effective tool for the identification of breast cancer metastases in axillary SLN that are not subjected to a very extensive histopathologic examination remains to be evaluated.

Epilogue

This review focused on some controversial issues of the histopathologic assessment of SLN status, with special reference to the assumption that the detection of even minimal lymph node involvement may be clinically relevant, both for immediate surgical intervention on further-echelon lymph nodes and for prognostic/predictive evaluations.

The mere occurrence of tumour cells in the regional lymph nodes, however, may not be an invariable predictor of clinical progression of the disease. The results of randomised trials [43, 44, 50] have shown that the observed number of breast carcinoma patients with clinically overt axillary

progression of the disease is much lower than expected based on either the false-negative rate of the sentinel lymph node biopsy or the known prevalence of metastasis to axillary lymph nodes. This suggests that metastatic cells may not progress to clinical disease in all patients and that only some of them have the capability of sustaining tumour progression, consistent with the hypothesis that cancer growth and progression, and hence the clinical outcome of the disease, are dependent on the activation of tumorigenic stem/progenitor breast cancer cells [1, 12].

The stem/progenitor cell theory of carcinogenesis and tumour progression conflicts with the traditional stochastic approach, by which all tumour cells in a given patient share the same potential for progression. According to the stochastic approach, prognosis is dictated by the actual amount of invasive or metastatic tumour cells, and the therapeutic interventions are intended to minimize their number. This leads pathologists to evaluate painstakingly tumour burden and count tumour cells, using all the possible ancillary techniques to identify even individual tumour cells. This attitude may not be truly rewarding. Indeed, the progenitor/stem cell theory predicts that only some (and possibly a minor percentage of) tumour cells are actually responsible for tumour progression and clinical outcome, and that all efforts should be made to target these cells with specific interventions [16]. Thus, what is now needed is to unveil specific markers of the tumorigenic cells that would allow their identification and quantitation in clinical specimens and to re-evaluate the prognosis by alternative means.

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