Review

Intraoperative assessment of sentinel lymph nodes in breast cancer

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Background: **Sentinel lymph node biopsy (SLNB) reduces the morbidity of axillary clearance and is the standard of care for patients with clinically node-negative breast cancer. The ability to analyse the sentinel node during surgery enables a decision to be made whether to proceed to full axillary clearance during primary surgery, thus avoiding a second procedure in node-positive patients.**
Methods: **Current evidence for intraoperative sentinel node analysis following SLNB in breast cancer was reviewed and evaluated, based on articles obtained from a MEDLINE search using the terms 'sentinel node', 'intra-operative' and 'breast cancer'.**
Results and conclusion: **Current methods for evaluating the sentinel node during surgery include cytological and histological techniques. Newer quantitative molecular assays have been the subject of much recent clinical research. Pathological techniques of intraoperative SLNB analysis such as touch imprint cytology and frozen section have a high specificity, but a lower and more variably reported sensitivity. Molecular techniques are potentially able to sample a greater proportion of the sentinel node, and could have higher sensitivity.**

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Introduction

Completion of the NEW START sentinel lymph node training programme in December 2008[1] has allowed sentinel lymph node biopsy (SLNB) to become standard practice in the UK for early node-negative breast cancer, as recommended by current National Institute for Health and Clinical Excellence guidelines[2]. This change in the staging and management of the axilla means that approximately 25 000 women each year are spared more extensive axillary surgery[1].

The drive for less invasive management of the breast and axilla followed the success of national screening programmes in identifying breast cancer at an earlier stage. Less radical treatment of the breast in these patients was possible without detriment to long-term outcome. Similarly, less invasive management of the axilla was proposed in selected patients to avoid the morbidity of axillary clearance.

The histological status of the sentinel lymph node accurately reflects the overall status of the axilla in 97 per cent of cases[3–7]. Furthermore, avoidance of full axillary clearance on the basis of sentinel node staging does not increase the likelihood of axillary recurrence[8–10]. The Axillary Lymphatic Mapping Against Nodal Axillary Clearance (ALMANAC) trial compared 1031 patients with clinically node-negative breast cancer randomly assigned to one of two treatment pathways: 516 received primary axillary clearance or axillary sampling and 515 underwent SLNB with a delayed clearance or radiotherapy to the axilla when biopsy indicated nodal spread[11]. The trial demonstrated significantly reduced rates of lymphoedema and neuropathy, improved functional outcome and reduced hospital stay in the SLNB group, without a negative impact on patients' anxiety levels.

However, 25–30 per cent of patients undergoing SLNB will have a positive finding on biopsy[5,11]. Delayed axillary clearance as a second procedure following SLNB increases operating time and the duration of hospital stay[12]. This impact on bed occupancy and other health economic factors has driven research into intraoperative techniques

for evaluating the status of sentinel lymph nodes. Accurate intraoperative detection of sentinel node metastasis would allow axillary clearance to be undertaken immediately during the primary procedure when the sentinel node is involved, thereby avoiding a second hospital admission and general anaesthetic.

In 1999 the College of American Pathologists recommended the use of cytological methods to evaluate the sentinel node during surgery[13]. Since then, a plethora of research has been published on the use of histological, cytological and molecular diagnostic assays in staging the sentinel node. Recent coverage in the popular press[14] and a UK National Health Service initiative to facilitate national adoption of molecular techniques for intraoperative sentinel node analysis[15] have raised the profile of this debate. The present paper reviews current evidence evaluating the efficacy of histological, cytological and molecular techniques.

Methods

The MEDLINE database was searched using the terms 'sentinel node', 'intra-operative' and 'breast cancer'. All abstracts from English language articles and foreign language articles available in a translated form were examined by a single reviewer. Papers detailing relevant experimental data were assessed for quality independently by two separate reviewers. All review articles, systematic reviews and meta-analyses were assessed, and references of such articles were searched for additional relevant papers.

Papers that outlined their methodology sufficiently to allow comparison were included; articles that failed to detail the sectioning procedure of both the experimental technique and permanent histological control were excluded. Values for accuracy, sensitivity and specificity are given on a per-patient basis (unless stated otherwise) for reasons of clarity, because this was the most universally adopted format of data reporting. Where sufficient data were reported in the articles, values specific to macrometastasis and micrometastasis were derived, if not directly quoted by the original paper.

Current practice

Variation in local histological practice makes comparison of research data from different centres problematic. A pan-European survey of current practice within 240 units processing sentinel node biopsies demonstrated 123 different protocols in use[16]. Intraoperative assessment of SLNB was performed in 145 units (60·4 per cent). Of these, 101 (69·7 per cent) used frozen section in isolation, with a further 28 units employing a combination of imprint cytology and frozen section. Only 11·0 per cent of units used imprint cytology alone. Intraoperative immunohistochemistry (IHC) was performed in 9·7 per cent of laboratories. Further inconsistency was noted in the number of levels examined during surgery, with approximately 50 per cent of centres analysing a single level and 50 per cent examining multiple levels.

Variation in the reporting categories of lymph node metastasis size adds further complexity. For the purpose of this review, the following terms are used, as defined by current American Joint Committee on Cancer tumour node metastasis staging guidelines[17]: isolated tumour cells, single cells or small clusters of cells no greater than 0·2 mm in largest dimension; micrometastasis, tumour deposits larger than 0·2 mm but smaller than 2 mm in largest dimension; macrometastasis, tumour deposits greater than 2 mm in largest dimension.

Histological and cytological techniques

Current protocols employ frozen section, imprint and scrape cytology, rapid immunocytochemistry and combinations thereof in the intraoperative evaluation of sentinel nodes[16].

Frozen section

Frozen section is the most commonly used technique. Its reported sensitivity in published literature ranges from 57 to 74 per cent[18–24]. Protocols for intraoperative frozen-section analysis and formalin-fixed paraffin-embedded ('permanent') sectioning vary widely, making comparison between studies difficult. *Table 1* details the outcome of studies where comparison was made between frozen section and final histological staging of the sentinel node; studies where detail on protocol used was incomplete have been excluded.

Predictably, when frozen section is compared with formal histology, greater concordance is reported by studies where the protocol for frozen section involves more extensive examination of the node. The specificity reported in all studies consistently approached 100 per cent, indicating that, despite variation in reported false-negative rates, the false-positive rate with frozen section is close to zero.

Frozen section is expensive, labour intensive and operator dependent, requiring a skilled biomedical scientist and dedicated histopathologist for each surgical session. Frozen sections are morphologically inferior to paraffin sections (*Fig. 1*) and may miss subtle lymph node

Table 1 Published studies on the use of intraoperative frozen-section analysis of sentinel lymph node biopsies where number of levels examined in intraoperative and permanent histology was specified in methodology

Reference	No. of patients	SLNB-guided AXCL?	No. of SLNs examined	Frozen-section methods	Permanent staining methods	Accuracy (%)	Sensitivity (%)	Specificity (%)
Veronesi *et al*.[18] (1997)	107	All patients treated with AXCL	NS	SLN bisected if > 5 mm; 3 levels from one half; H&E stain	Paraffin; 3 levels from one half; H&E stain	Total 83	Total 64	Total 100
Weiser *et al*.[19] (2000)	890	Intraoperative, SLNB guided	NS	Single level; H&E stain	Paraffin; half node section at 50 μm; 3 sections H&E stain and 2 sections IHC (CAM5·2 AE1/AE3)	Total 89	Total 58 Macro 92 Micro 17	Total 99
Rahusen *et al*.[20] (2000)	100	Intraoperative, SLNB guided	160	SLN bisected if < 10 mm; if > 10 mm, 5-mm sections; single level from each section	Paraffin; initial single level; if negative, additional 4 levels; H&E stain; IHC (CAM5·2)	Total 85	Total 57 Macro 84 Micro 27	Total 100
Zurrida *et al*.[21] (2000)	192	All patients treated with AXCL	NS	Bisected; 3 levels taken from one half	Paraffin; 3 levels from each half; H&E stain	Total 86	Total 68	Total 100
Tanis *et al*.[22] (2001)	262	Intraoperative, SLNB guided	444	Bisected; single level; H&E stain	Paraffin; H&E stain from 3 levels; IHC from 1 level (CAM5·2)	Total 90	Total 74	Total 99
Van de Vrande *et al*.[23] (2009)	615	Intraoperative, SLNB guided	994	SLN bisected if > 5 mm; single level from one half; H&E stain	Paraffin serial section at 150 μm; H&E stain; IHC (CK-8)	Total 90·7	Total 71·6 Macro 84·0 Micro 61·1	Total 100
Viale *et al*.[24] (1999)	155	All patients treated with AXCL	203	Serial sections at 50-μm intervals; H&E stain and IHC*	None	NA	NA	NA

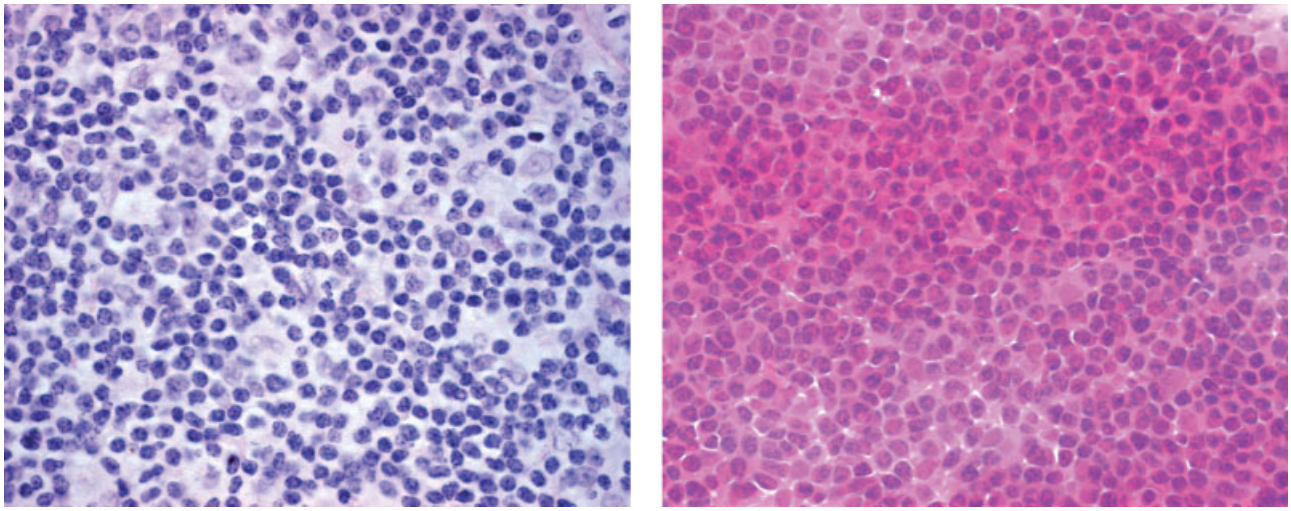
SLN(B), sentinel lymph node (biopsy); AXCL, axillary clearance; NS, not specified; H&E, haematoxylin and eosin; IHC, immunohistochemistry; macro, macrometastases; micro, micrometastases; CK, cytokeratin; NA, not applicable. *IHC methodology: rapid staining (EPOS anti-cytokeratin/HRP; Dako, Copenhagen, Denmark) with MNF116 monoclonal antibody.

metastases, particularly in lobular carcinoma, where the cells are usually cytologically bland and have an infiltrative growth pattern (*Fig. 2*). Furthermore, the process of cutting a frozen section results in irreversible tissue loss. Therefore, there is a theoretical potential for understaging of sentinel nodes when evidence of micrometastatic disease is corrupted by the frozen-section process. Unfortunately it is impossible to determine accurately the frequency of such an error through direct comparison. These problems with frozen section make an alternative desirable.

Intraoperative cytology

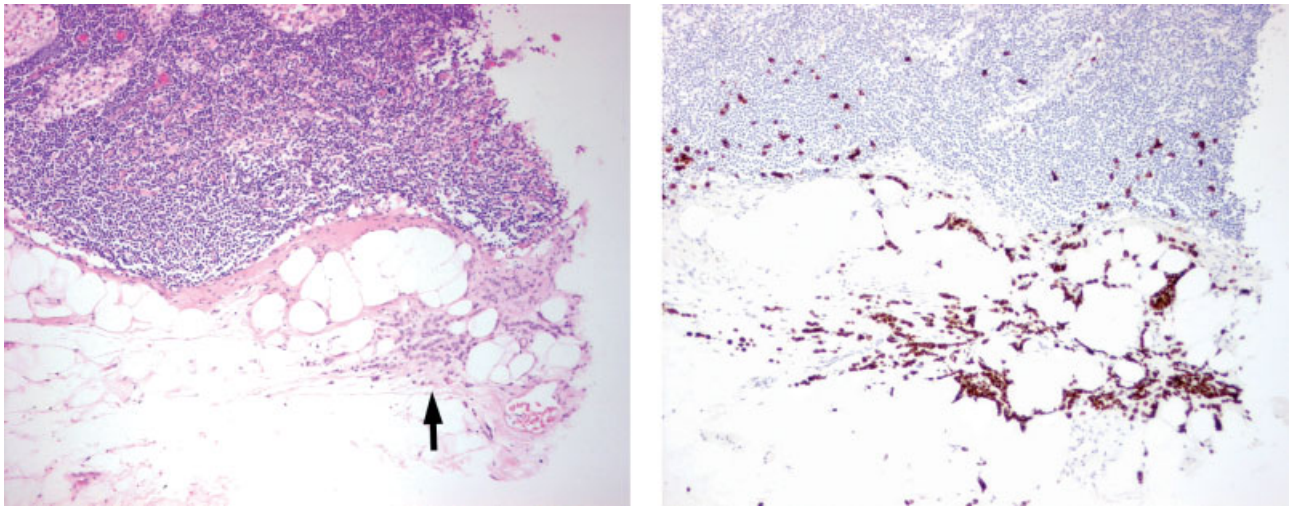
Cytological techniques such as intraoperative imprint and scrape cytology have some technical advantages over frozen-section analysis. The cut surface of the sentinel node is pressed or scraped on to a glass slide, stained and examined. The preparation time and cost of cytological specimens is less than for frozen section, and there is no loss of tissue. Disadvantages include the small number of cells analysed, the significant expertise required to interpret cytological material and the potential for an inconclusive report that fails to guide intraoperative decisions.

In 2005, Tew and colleagues[25] published a meta-analysis of 31 articles on the use of touch imprint cytology in sentinel node staging. Heterogeneity of methodology again makes these data difficult to interpret; within the 31 studies, there were differences in intraoperative assessment (6 different techniques), sectioning method (11 distinct protocols), imprint staining used (9 different stains used in various combinations), application of rapid IHC and immunofluorescence techniques (used in 7 of the 31 studies) and final staining method (3 distinct protocols). A random-effects model pooled estimate of the sensitivity of imprint cytology was 63 (95 per cent confidence interval (c.i.) 57 to 69) per cent and the specificity was 99 (98 to 99) per cent.



a Conventional paraffin section of lymph node **b** Frozen section of lymph node

Fig. 1 Comparison of the quality of paraffin and frozen sections. **a** Conventional paraffin section and **b** frozen section of the same lymph node (haematoxylin and eosin stain, original magnification × 600). The nuclear and cytoplasmic detail is seen more clearly on the paraffin section. The nuclear detail is obscured in the frozen section and the cytoplasm appears abnormally prominent



a Haematoxylin and eosin staining of metastasis **b** Cytokeratin immunohistochemistry of metastasis

Fig. 2 Lymph node containing a subtle metastasis from a lobular carcinoma. **a** On haematoxylin and eosin staining (original magnification × 100), an infiltrate of small, bland cells can be seen in the extranodal fat (arrow). **b** Cytokeratin immunohistochemistry (MNF116 × 100) highlights the more extensive nature of the metastasis, with single cells seen infiltrating into the node and more widely in the extranodal fat. It is highly likely that this subtle metastasis would have been missed on frozen section

A significant variation continues to exist in the reported sensitivity of cytological techniques. Since the publication of Tew and co-workers[25], further studies of imprint cytology have been published. *Table 2* details their methods and results, with sensitivity ranging from 33 to 73 per cent and specificity of 98–100 per cent[25–29].

False-negative results in imprint cytology are more common in the presence of micrometastatic disease[25,30] and in invasive lobular carcinoma[28]. Tew *et al.*[25] estimated that imprint cytology detected macrometastasis in SLNB with 81 per cent sensitivity and micrometastasis with 22 per cent sensitivity. The size of micrometastasis and the small amount of cellular tissue examined combine to make

Table 2 Use of imprint cytology to stage sentinel nodes. Summary data from Tew *et al.*[25] alongside papers not incorporated in the meta-analysis

	No. of patients	Touch imprint methods	Permanent section methods	Accuracy (%)	Sensitivity (%)	Specificity (%)
Pooled data from 31 studies included in Tew *et al.*[25]	4438	Various	Various		Total 63 Macro 81 Micro 22	Total 99
Barranger *et al.*[26]	180	Bisected; Diff-Quick stain	3-mm sections, each analysed 4 times; 150-μm levels; H&E + IHC (AE1–AE3)	Total 79	Total 33 Macro 75	Total 98
Chicken *et al.*[27]	133	Bisected; Giemsa stain	Sections at 3 levels; H&E + IHC (AE1/AE3)	Total 95	Total 73	Total 100
Cox *et al.*[28]	2137	Bisected; Diff-Quick stain	Single section; further sections taken if initial section negative; H&E + IHC (CK)	Total 85	Total 53 Macro 69·3 Micro 6·4	Total 99
Contractor *et al.*[29]	896	Bisected; H&E stain	Single section; H&E stain	Total 92·5	Total 73	Total 100

Macro, macrometastasis; micro, micrometastasis; H&E, haematoxylin and eosin; IHC, immunohistochemistry; CK, cytokeratin.

their detection difficult by imprint cytology. Increasing the number of cut nodal surfaces sampled would increase the pick-up rate simply by increasing the total volume of the node examined. However, studies in which each node is sectioned extensively once formalin fixed and paraffin embedded are likely to find a lower sensitivity for imprint cytology as they identify micrometastatic disease with greater frequency during final histological examination.

Invasive lobular carcinoma presents an additional problem because its cells are usually of low histological grade, are poorly cohesive and may resemble lymphoid cells morphologically (*Fig. 3*). This makes their detection on cytological specimens difficult. Cox and colleagues[28] reported a sensitivity of 38·7 per cent in identification of lobular carcinoma metastasis using imprint cytology, compared with 55·5 per cent in invasive ductal carcinoma.

Some authors have advocated the routine use of immunocytochemical techniques in intraoperative imprint cytology of specimens from patients with known invasive lobular carcinoma after demonstrating that this technique improves diagnosis markedly[31]. Similarly, the use of immunocytochemical techniques has also been demonstrated by some authors to improve the detection of micrometastasis on imprint slides[32]. In the meta-analysis by Tew *et al.*[25], the pooled estimate of sensitivity in the seven studies that employed immunocytochemistry was 66 per cent, compared with a pooled sensitivity of 60 per cent in studies where immunocytochemistry was not used[25]. However, immunostaining has an uncertain role in intraoperative staging of SLNB; it is time consuming and expensive, making it less practical for intraoperative use.

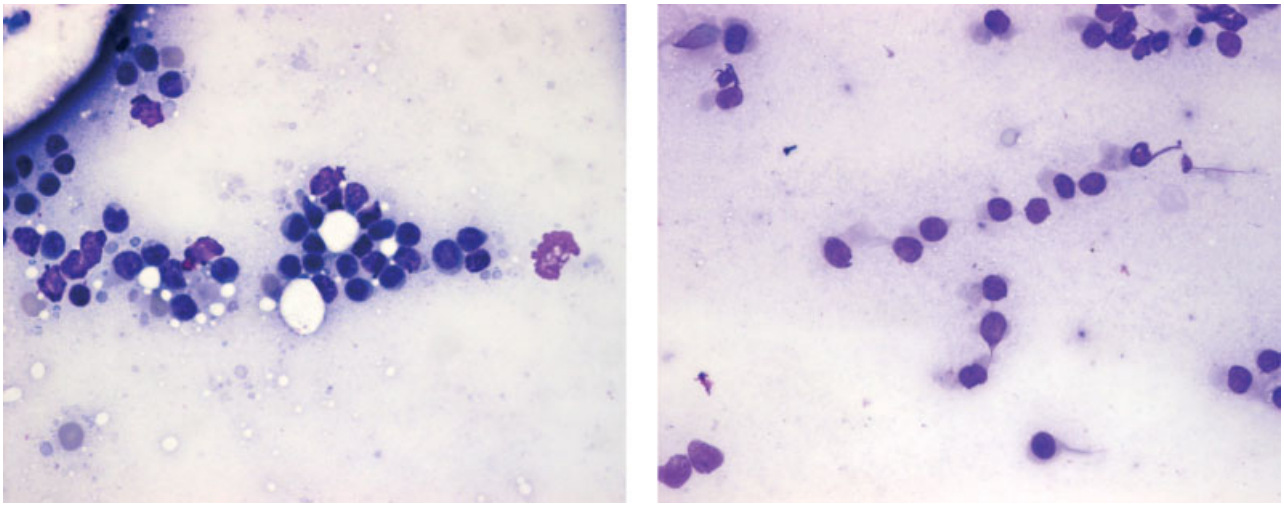
Frozen section *versus* intraoperative cytology

Three of four studies[33–36] comparing frozen section and imprint cytology found frozen section to have a greater sensitivity than imprint cytology[33–35] (*Fig. 4*). The fourth study[36] employed immunostaining and showed an advantage in the use of imprint cytology. Tew and co-workers[25] estimated pooled sensitivity and specificity for frozen section to be 76 and 99 per cent respectively, compared with 63 and 99 per cent for imprint cytology. The small advantage reported in sensitivity for frozen section might well be overcome by increasing the number of slides taken during imprint cytology. Such an increase would improve sensitivity[37] without the deleterious effect of losing tissue for formal histological examination, which remains the key advantage of imprint cytology over frozen section.

Molecular techniques: quantitative reverse transcriptase–polymerase chain reaction and one-step nucleic acid amplification

Standard histological sampling protocols examine only a small proportion of the total volume of the sentinel node. This introduces the probability of significant sampling error in these techniques: a negative result might occur simply through failure to examine the part of the node that contains metastasis.

Molecular techniques have the potential to eliminate sampling error. The sample tissue is homogenized and scrutinized for the presence of marker genetic material. This potentially enables analysis of the entire node. As these techniques require the presence of only a single trained technician at the point of analysis, this increase in



a Giemsa staining of lymphocytes **b** Giemsa staining of lobular carcinoma cells

Fig. 3 Comparison of **a** lymphocytes and **b** lobular carcinoma cells (both Giemsa stain, original magnification × 400). Both cell types have small, round, bland nuclei. The lobular carcinoma cells are subtly different, possessing more cytoplasm and having eccentrically located nuclei

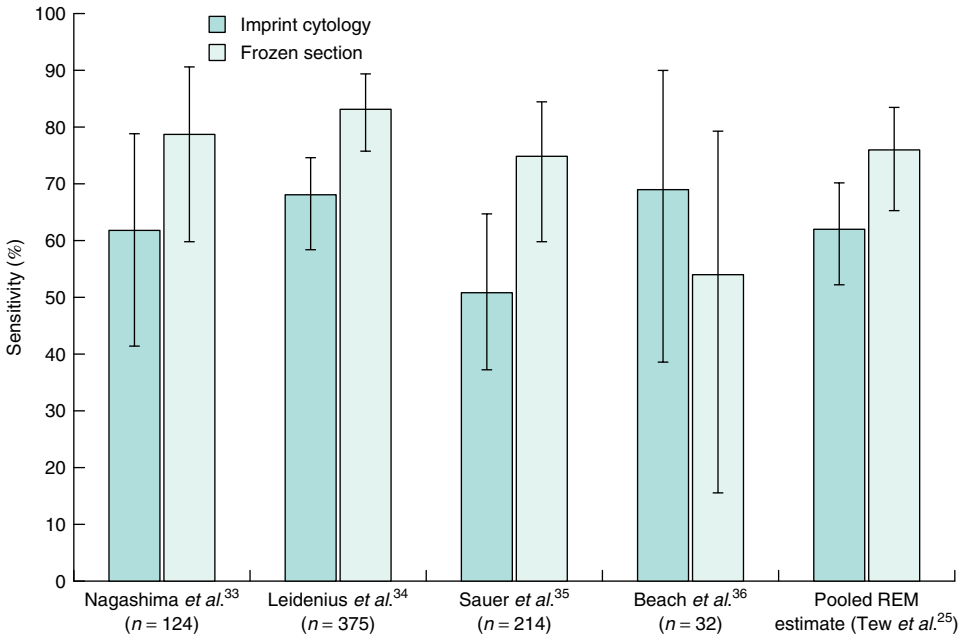


Fig. 4 Results of four studies comparing the sensitivity of frozen section with that of imprint cytology. Error bars denote 95 per cent confidence intervals. REM, random effects model

volume examined is attainable without greatly increasing the burden on the histopathology department.

Both quantitative reverse transcriptase–polymerase chain reaction (qRT–PCR) and one-step nucleic acid amplification (OSNA) have been proposed as viable techniques for intraoperative node analysis. They rely on detection of the mRNA for marker genes that are overexpressed in tumour cells but not in normal tissue.

Quantitative reverse transcriptase–polymerase chain reaction

Molecular diagnostics was proposed initially as a method for detecting tumour-specific antigens in peripheral blood, lymphatic tissue and bone marrow[38–42]. The presence of such antigens remains of uncertain prognostic significance but is the subject of ongoing research[43,44]. The value of molecular assays in the detection of lymph node metastasis was limited by the high sensitivity of the techniques; tumour mRNA markers, although expressed in neoplastic cells, are also present in normal tissues, albeit to a lesser extent[45]. Specificity was therefore too low to be of use in detecting metastases with qualitative techniques[46–48].

Newer quantitative techniques, such as qRT–PCR, allow differentiation between the high levels of marker mRNA expressed by tumour cells and the low, legitimate expression by non-neoplastic tissues[49,50]. These techniques use fluorescence to calculate the quantity of target genetic material produced real-time during PCR. This is compared to a threshold level – the level that would be the upper limit of normal expression within non-neoplastic tissues. An expression above the threshold indicates the presence of metastasis.

The ideal marker for detection of sentinel node metastases would be expressed by 100 per cent of metastatic breast cancer cells but not by any non-neoplastic tissues, and be suitable for DNA probe design. Although some genes, such as those for cytokeratin (CK) 19 and mammaglobin (MGB) 1, are expressed by the vast majority of breast cancers, no one gene is expressed universally. This therefore limits the sensitivity of single-marker assays[51]. It is agreed that multigene assays increase sensitivity[52,53]; however, the use of too many markers might have a deleterious effect on the specificity of the assay.

The optimal number of markers is probably two or three, although which genes should be used remains controversial. Backus and colleagues[52] compared molecular techniques with extensive histological sectioning under laboratory conditions. They achieved 91 per cent sensitivity and 97 per cent specificity using a combination of MGB1 and CK-19 markers. Hughes and co-workers[53] estimated sensitivity in excess of 97 per cent when using either MGB1 and CK-19, or prolactin-inducible protein (PIP) and tumour-associated calcium signal transducer 1 markers[53]. However, three pseudogenes for CK-19 exist within the human genome, causing concern that, if RNA isolation is not complete before PCR, false positives with CK-19-based assays are a possibility[53].

The first commercially available qRT–PCR assay for intraoperative assessment of sentinel node material was the GeneSearch™ Breast Lymph Node (BLN) Assay (Veridex, Warren, New Jersey, USA). The assay kit provides standard reagents, controls and detailed protocols, which allow maximum reproducibility within and between laboratories. It relies on the use of MGB1 and CK-19 in a dual-marker assay. A positive assay is one where the expression of either marker exceeds a threshold level, calibrated to correlate with the presence of metastases greater than 0·2 mm in diameter. The implementation of such technology has, however, been hindered by the imminent withdrawal of the commercial GeneSearch™ BLN assay. Reasons suggested include poorer than expected uptake in the USA, particularly in centres already running intraoperative pathological analysis such as frozen section, high start-up costs and continued uncertainty regarding the significance or otherwise of the low experimental specificity when compared to histological sectioning[54]. Despite this, the principle of molecular analysis through qRT–PCR techniques has been established, and work is under way on developing non-commercial open-access alternatives.

Eight papers have been published evaluating the application of qRT–PCR, with promising results (*Table 3*)[55–62]. The overall sensitivity of qRT–PCR was 78–96 per cent, exceeding that of imprint cytology and frozen section. Julian *et al.*[55] directly compared qRT–PCR with frozen section in 319 patients and found a sensitivity (95 per cent c.i.) of 95·6 (89·0 to 98·8) and 85·6 (76·6 to 92·1) per cent respectively when using permanent histological sectioning as standard. qRT–PCR also appears to detect metastatic lobular carcinoma more effectively than histological techniques[56].

All studies compared molecular analysis with extensive sectioning. Unfortunately, because qRT–PCR requires homogenization of sample tissue, histological examination of the same tissue, and therefore direct comparison, was not possible. This leaves the potential for discrepancies due to sampling error. Such error may account for the lower specificity seen in qRT–PCR. False-positive results in qRT–PCR may occur when the metastatic deposit is entirely within that part of the node undergoing qRT–PCR analysis, so that it remains undetected by histological techniques. The converse may also, of course, be true, with sampling error erroneously reducing the apparent sensitivity of qRT–PCR.

Despite the undoubted significance of this sampling effect, the apparent lower specificity of molecular techniques does raise the question whether such assays are prone to true false-positive results. Further analysis

Table 3 Performance of quantitative reverse transcriptase–polymerase chain reaction in published literature

	No. of patients	Subgroup analysis	Sensitivity (%)*	Specificity (%)*	Agreement (%)
Julian *et al.*[55] and Blumencranz *et al.*[56]†	416	Total 416	87·6 (80·4, 92·9)	94·2 (90·9, 96·6)	92·3
		Macro 94	97·9 (92·5, 99·7)		
		Micro 23	56·5 (34·5, 76·8)		
		Lobular 57	80·0	91·9	87·7
Viale *et al.*[57]	293	Total 293	77·8	95·0	90·8
		Macro 52	98·1		
		Micro 20	25		
Martinez *et al.*[58]	82	Total 124	88·9 (56·5, 98·0)	95·7 (90·2, 98·1)	95·2
		Macro 6	100		
		Micro 3	66·7		
Mansel *et al.*[59]	78	Total 78	92	97	96
Veys *et al.*[60]	367	Total 367	89	94·5	93·5
Tafe *et al.*[61]	59	Total 59	88·9 (51·8, 99·7)	93·5 (82·1, 98·6)	86·4
Cutress *et al.*[62]‡	254	Total 256	96	95	95
		Macro	100		

*Values in parentheses are 95 per cent confidence intervals. †References 55 and 56 grouped together because they involved the same patient group and only data from the validation cohort in each study were described; ‡data compared reverse transcriptase–polymerase chain reaction (RT–PCR) result with final axillary node status, which took into account non-sentinel nodes in cases where intraoperative RT–PCR was positive. For all other references, data represent comparison of sentinel node status in RT–PCR *versus* histopathology. Macro, macrometastases; micro; micrometastases.

of lysate from BLN-positive, histology-negative tumours with additional molecular markers B305D, B726, PIP and prostate-derived Ets transcription factor supported the presence of metastatic material in 73–76 per cent of these samples[55,56]. This suggests that the reported specificity was an underestimate of true specificity owing to histology sampling a smaller proportion of the node than the molecular assay.

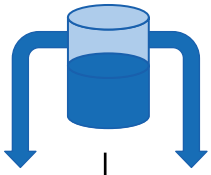
In addition, each assay is run with a series of internal and external controls to protect from operator error or kit dysfunction producing false-positive or false-negative results. There does, however, remain a potential for contamination of node samples with breast tissue, which would result in false-positive assays. Rigorous surgical technique and minimizing the amount of extranodal tissue homogenized during sample preparation are necessary to reduce the risk of such contamination.

One-step nucleic acid amplification

OSNA, like qRT–PCR, is a molecular diagnostic technique used to detect target gene mRNA. It also uses reverse transcriptase to convert mRNA to cDNA; however, gene replication is by loop-mediated isothermal amplification (LAMP). This variation on PCR uses six primers specific to the same cDNA target. These primers are designed so that looping of the DNA occurs during the amplification phase. This releases pyrophosphate as a byproduct, which binds with magnesium and precipitates. The rate of precipitation, or turbidity, of the solution is used to quantify the amount of target gene present[63].

The difference between RT–LAMP (OSNA) and qRT–PCR is that OSNA does not use the denaturation steps required in qRT–PCR (*Fig. 5*). In addition, because of the pH and temperature at which OSNA is run, there is no need for meticulous extraction of RNA from genomic DNA. The lysate is buffered at pH 3·5, which precipitates the vast majority of genomic DNA, and isothermal cycling at 65°C is too cool for genomic DNA to denature. Therefore, only cDNA is available for the primers to bind to. The fact that six primers are required to bind the same gene also increases the assay's specificity. This means that OSNA is relatively immune from genomic pseudogene interference which is, as discussed above, a possible source of false-positive results in qRT–PCR, particularly for CK-19[53].

Tsujimoto and colleagues[65] recently described a protocol for the use of OSNA in the detection of CK-19 mRNA within sentinel nodes. Concordance of extensive three-level histology with 2-mm sectioning, using IHC stains for CK-19, was 98·2 per cent. The assay also showed some ability to differentiate macrometastasis from micrometastasis. The same protocol was ratified by an independent group of researchers who examined 346 stored axillary nodes[64]. These authors demonstrated a sensitivity of 95·3 per cent and a specificity of 94·7 per cent for OSNA CK-19 using histology as a comparison (serial



qRT–PCR

1. mRNA isolation (various)
2. Production of cDNA by reverse transcriptase
3. Thermal cycling with DNA polymerase and primer specific to cDNA of gene
   (a) 2 min at 50°C
   (b) 10 min at 95°C
   (c) 40 cycles of:
      I. 15 s at 95°C
      II. 1 min at 60°C

OSNA (RT–LAMP)

Addition of reagent including:
- Buffer (pH 3·5)
- Reverse transcriptase
- 6 primers specific to cDNA of gene
- DNA polymerase

Held at constant temperature of 65°C

	qRT–PCR	OSNA
Time for assay	26–43 min[59]	30–40 min[64]
Isolation of RNA	Various	None
Thermal cycles	40	None (isothermic)
Primers	1	6
Quantification	Fluorescence (4 techniques)	Turbidity
Automation	Full	Full

Fig. 5 Methodology of quantitative reverse transcriptase–polymerase chain reaction (qRT–PCR) and one-step nucleic acid amplification (OSNA). SLNB, sentinel lymph node biopsy; RT–LAMP, reverse transcriptase–loop-mediated isothermal amplification

sectioning at 250-μm intervals; haematoxylin and eosin with CAM5·2 IHC staining)[64]. Both authors described the effect of sample error when comparing OSNA with histological sectioning.

Schem *et al.*[66] examined 343 non-sentinel lymph nodes from patients undergoing completion axillary clearance who were identified before surgery as being node positive clinically or following a positive SLNB. The nodes were split into four sections and comparison was made between OSNA CK-19 and five-level histological sectioning with CK-19 and Lu5 IHC staining. Reported sensitivity on a per-node basis was 98·1 per cent; however, per-node specificity within the entire cohort was 89·0 per cent. When qRT–PCR and western blot techniques were carried out on the remaining lysate from OSNA-positive, histology-negative tumours, 11 of 26 samples were positive for markers of metastasis, suggesting that 89 per cent was an underestimate of the true specificity.

The results from a further Japanese multicentre study support this, demonstrating a specificity of 97·1 per cent (95 per cent c.i. 91·8 to 99·4) from 124 axillary nodes where OSNA was compared with extensive sectioning at 0·2 mm intervals with HE staining and IHC for CK-19[79]. However, when performance of OSNA was evaluated with a further 450 axillary nodes, using a "routine" histological sectioning protocol as comparison (three sections taken from the cut surface of the quartered node) the sensitivity was 87·7 per cent (95 per cent c.i. 78·5 to 93·9); overall agreement was 92·9 per cent (95 per cent c.i. 90·1 to 95·1).

Additional clinical studies are required to evaluate this emerging technique further.

Future methods

Elastic scattering spectroscopy (ESS) detects the abnormal cellular architecture present in metastatic disease through changes in light absorption and scattering properties. A probe interrogates tissues by emitting pulses of white light and collecting the backscattered signal. A computer then analyses the return signal for changes characteristic of tightly packed cellular constituents (nucleus, mitochondria) or abnormal relative size of these components.

Such probes are able to interrogate a volume of tissue 0·5 mm in diameter and 1 mm deep with each flash of light. This technique has been used in Barrett's oesophagus to differentiate between normal tissue, high-grade dysplasia and carcinoma[67], and in SLNB to detect breast cancer metastasis[68]. This technology remains experimental and the first clinical trial results are just starting to be reported[78].

ESS offers the possibility of intraoperative analysis of the sentinel node without the need for a specialist pathologist. Other potential advantages include minimal tissue preparation and destruction, instant results and low running costs. However, because the device can only analyse tissue of a maximal thickness of 1 mm, the same confounding sampling errors implicit in sectioning will apply as for histological analysis.

Discussion

A problem common to all histological methods of intraoperative staging is that any protocol used is a compromise

between sensitivity and practicality. Comprehensive evaluation of a 2-cm node aimed at finding all metastatic disease more than 0·2 mm in size would require 100 sections. Viale *et al.*[24] have described a protocol whereby the entire node is subjected to frozen section, with over 60 sections taken from each node. However, application of this technique to intraoperative analysis required a team of histopathologists in theatre to analyse material[69], which is clearly beyond the means of most centres.

As qRT–PCR has the potential to reduce or eliminate sampling error, depending on the amount of tissue reserved for histological examination, it may provide a more sensitive assessment of the sentinel node than histology alone. A study by Weigelt and colleagues[70] analysed 70 sentinel nodes staged as negative for metastasis by conventional histology. The qRT–PCR assay identified seven nodes as positive, four of which were found to contain micrometastases on further histological examination.

There is, however, an inherent error in attempting to validate molecular assays through comparison with histopathology; the tissue for qRT–PCR is homogenized and is therefore not available for histological examination. The two techniques never examine the same tissue and discrepancies due to sampling will occur. Similar discrepancies have been shown to occur in histological examination: 6 per cent of histological slides will be negative despite sections from adjacent tissue being positive[55]. Investigators have therefore argued that a 94 per cent concordance between molecular assays and histology is the maximum expected, the 6 per cent discrepancy in results being accounted for by sampling error.

Existing data suggest that molecular assays are more sensitive than frozen section and imprint cytology for the intraoperative analysis of sentinel lymph nodes. By identifying a higher proportion of sentinel node metastases, molecular assays would prevent a greater number of secondary axillary clearances. Cost analysis performed at a large UK district general hospital found that savings implicit in reducing numbers of secondary procedures, such as reduced bed and theatre occupancy, comfortably offset the expense of intraoperative RT–PCR for the health economy, although current tariff structures reduce the attractiveness to individual hospitals[62].

Although molecular assays may potentially mitigate the strain on pathology services implicit in the introduction of intraoperative sentinel node assessment techniques, increased intraoperative sentinel node analysis may lead to difficulties in theatre scheduling. The impact would be minimized by increased preoperative axillary screening with ultrasonography and fine-needle aspiration, allowing node-positive patients to proceed directly to immediate axillary clearance. Stratification of clinically and radiologically node-negative axillae within theatre lists by criteria such as tumour size and grade, which are known to be predictive of the probability of node positivity[71], might minimize the likelihood of the majority of patients on a single operating list requiring conversion to axillary clearance.

Clear explanation and adequate preoperative counselling undoubtedly play a vital role in the implementation of intraoperative SLNB analysis. The psychological effect on patients who undergo SLNB with preoperative uncertainty as to whether they will proceed to an axillary clearance merits further study, as does the impact of the small proportion of false-positive or false-negative intraoperative results.

It has been suggested that there is potential for molecular techniques to supplant formal histology as the standard method for detection of metastasis. Advantages include greater automation, analysis of a greater volume of the lymph node, the rapidity of such tests, financial savings and the objective nature of molecular diagnostics. The importance of objectivity should not be underestimated. Discordance between pathologists in the interpretation of slides can be considerable; one study showed that when ten independent pathologists looked at slides taken from sentinel node biopsies, 100 per cent agreement in interpretation occurred in just 12 per cent of cases[72].

When using molecular techniques exclusively, histopathological markers of prognosis such as size of metastatic deposits and presence of extranodal or extracapsular spread would remain unrecognized. Loss of such important indicators, which are widely used to guide contemporary oncological practice, is a significant disadvantage. Furthermore, storage of histological samples allows cases to be reviewed years after the index presentation. Often only the histological features of the index primary metastasis can be used to differentiate between recurrence and a new focus of primary disease.

Introduction of molecular diagnostic techniques into clinical practice would increase the number of positive lymph node biopsies. Weigelt and co-workers[70] suggested that RT–PCR might upstage at least 10 per cent of sentinel nodes, subsequently increasing the number of axillary clearances performed, yet the benefit of axillary clearance in patients with low-volume metastatic disease is unclear. The incidence of non-sentinel node disease is far greater in the presence of macrometastasis (63 per cent) compared with that present with micrometastases[73]. Meta-analysis of the reported incidence of non-sentinel node involvement in the presence of isolated micrometastasis within the

sentinel node is 10–15 per cent, and falls to 9 per cent when sentinel node disease is only identifiable when IHC is used[74]. The presence of micrometastatic disease is generally considered a negative prognostic indicator[75,76], although this remains controversial[77] and not all studies have shown prognostic significance.

Intraoperative analysis of SLNB continues to evolve, while its application becomes more widespread. The question remains which technique will dominate future practice? Molecular-based techniques offer the greatest propensity for intraoperative diagnosis of low-volume metastatic disease, appearing to outperform histological techniques. They also provide an objective result quickly, are cost effective and do not invoke the expense of a dedicated pathologist. qRT–PCR techniques are also becoming increasingly prevalent in other areas of medicine, with the result that investment in equipment could be spread over several departments. This confers an advantage of qRT–PCR over OSNA which, at present, has far fewer additional clinical applications.

Although questions remain over the appropriate management of low-volume metastases within sentinel nodes, both qRT–PCR[60] and OSNA[65] are able to differentiate between micrometastatic and macrometastatic disease. Their use might therefore still be practical in centres where axillary dissection is reserved for patients with macrometastatic disease[59].

Whether quantification of molecular markers of tumour cell metastases, such as CK-19 and MGB1, within sentinel node and other tissues provides an independent prognostic indicator in patients with breast cancer remains unclear. The establishment of such a link between levels of molecular markers and disease progression might conceivably allow molecular diagnostics to supersede formal histopathology. In the immediate future, however, it is likely that the two techniques will continue to be applied simultaneously.

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