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REVIEW

Molecular assessment of sentinel lymph node in breast cancer management

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Sentinel lymph node biopsy (SLNB) is becoming accepted as standard of care for axillary staging in breast cancer. World Health Organization (WHO) reclassification of axillary metastases into macrometastases, micrometastases and individual tumour cells has highlighted the issues of sampling and further histological examination of the initially negative SLNB. Molecular detection of metastatic breast cancer cells in lymph nodes is now available as a commercial kit for intraoperative use and can resolve the sampling issue. Semiquantitative assessment of axillary lymph node tumour burden can now be made using two separate technologies (histology and reverse transcriptase–polymerase chain reaction). The clinical implications of low metastatic axillary lymph node tumour burden are not clear, and future trials need to include molecular data. The consequences of the availability of molecular assessment are reviewed.

Keywords: breast, intraoperative diagnosis, polymerase chain reaction, sentinel lymph node

Abbreviations: CK, cytokeratin; H&E, haematoxylin and eosin; ITC, individual tumour cells; MG, mammaglobin; Mi, micrometastasis; mRNA, messenger RNA; RT-PCR, reverse transcriptase-polymerase chain reaction; SLN, sentinel node; SLNB, sentinel lymph node biopsy

Background to sentinel lymph node biopsy

The sentinel node (SLN) is defined as the first node receiving lymph from an anatomical area. The sentinel lymph node biopsy (SLNB) technique using tracer injected around the primary tumour to identify the most likely lymph node to contain metastasis was pioneered in carcinoma of the penis and melanoma.[1–3] SLNB has been developed and accepted in breast cancer management in recent years, although involvement of non-SLNs and limited long-term follow-up data on patients treated by SLNB alone have raised concern.[4–7] To improve its reliability in breast, two tracers, a blue dye and a technetium-labelled nanocolloid, are injected peritumorally and intradermally prior to surgery.[8] At surgery one or more sentinel node(s) are identified in axillary fat as blue or radioactive ('hot') using dedicated directional probes. Nodes may be blue, hot or hot and blue or clinically suspicious at surgery. If no tumour is found in the first node-draining tumour, the chances of other draining axillary lymph nodes containing tumour are extremely small.[9] The successful implementation of the technique involves a multidisciplinary team including ward and theatre nurses, nuclear medicine personnel, surgeons and pathologists. The importance of training the team to perform the technique optimally has been recognized, and in the UK a national training programme has been developed.[10]

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Histopathological assessment of the SLN: micrometastasis and individual tumour cells

The sixth edition of the World Health Organization (WHO) staging for breast cancer introduces a semiquantitative assessment of nodal burden categorized as

individual tumour cells or groups of cells <0.2 mm (ITC), micrometastasis between 0.2 and 2 mm diameter (Mi) and macrometastasis >2 mm.[11–13]

Whereas prior to the SLNB pathologists were routinely presented with axillary clearance specimens in which any node might prove to contain metastatic carcinoma, they are now presented with a limited number (1–4) of lymph nodes with a higher index of suspicion for positivity, justifying a more thorough examination. This begs the question for pathologists examining SLN: how much is enough?[12,14] The chances of finding a single ITC in a 4 μm section of a 10 mm lymph node are 0.4%. In order to identify ITC histologically >300 sections would be required from a 10 mm node.[12,14] A survey of 240 European laboratories has shown 127 different protocols for the further investigation of an initially negative SLNB. Four percent of laboratories used molecular assessments, but these were not further defined.[15] Part of the problem is that the prognostic significance of ITCs and micrometastatic disease is unknown, and if there is prognostic significance it is likely to be small. Should a patient with individual tumour cells or micrometastatic disease in their axillary lymph node be offered axillary clearance? A meta-analysis by Cserni *et al.* has shown a 20% probability of non-SLNs being positive if the SLN contains low-volume disease (Mi or ITC) and 9% if the SLN metastasis is found by immunohistochemistry alone.[16] The risk of higher echelon metastases has been related to SLN metastasis size and shown to be 2.24 times higher if the SLN metastasis is >1 mm, where as with smaller SLN Mi the risk is no greater than with ITC.[17]

Are these metastases in non-SLNs of prognostic significance in these patients? They may not be viable or capable of further growth because of inability to induce angiogenesis or stromal support. Should these patients with ITC or micrometastatic disease in the axillary lymph nodes be offered adjuvant chemotherapy? As the biological and prognostic significance of these findings is unknown and there are no mature clinical trials to guide management, these questions are currently unanswerable.

Allied to these questions is whether immunohistochemistry for cytokeratin (CK)-positive cells should be used routinely to find small metastatic tumour deposits. Current guidelines suggest that routine use of immunohistochemistry to find occult micrometastatic disease or ITC is not justified, as the prognostic significance of this level of tumour metastasis is not established.[18–23] Some pathologists would argue that routine use of immunohistochemistry is justified to save time highlighting metastatic cells more efficiently.

Intraoperative assessment

If a patient undergoes surgical excision of the primary breast cancer and the SLN(s) are fixed and sent to the laboratory with the main specimen the patient will face a second operation for axillary clearance if a SLN is positive. The chances that a second operation will be required can be reduced by preoperative assessment of axillary nodes by imaging and biopsy, and those with positive lymph nodes will not be offered SLNB. Some success at preoperative staging has been reported with ultrasound, and it is claimed that ultrasmall particles of dextran-coated iron oxide-enhanced magnetic resonance imaging can achieve 100% correct preoperative staging of the axilla, although details of the thoroughness of histopathological examination are lacking.[24,25]

An alternative approach is intraoperative assessment of the SLNBs by imprint cytology, frozen section and smears with or without the use of rapid immunohistochemistry for epithelial markers that has been developed and used in this setting.[26–29] All these techniques producing a reliable result within an intraoperative time frame (usually around 40 min), often on multiple SLNs (2.2 on average in the Axillary Lymphatic Mapping Against Nodal Axillary Clearance trial), can cause logistical problems.[30] These intraoperative examinations sample only a fraction of the material available, and Veronesi reported a 24% false-negative rate for intraoperative frozen section at three levels.[6] These false negative cases cause particular problems, in that the patient wakes from anaesthesia with an intact axilla and patient and surgeon are reassured. However, in the false-negative situation a paraffin-based definitive report emerges later showing positivity in one or more sentinel nodes, and the surgeon has to go back to the patient with the news that a second clearance operation will be required after all. To address this problem extensive histological sampling has been developed, where the entire SLN is submitted for frozen section with two sections [for haematoxylin and eosin (H&E) and immunohistochemistry] every 50 μm[31] through the node resulting in 60 frozen sections per node. If still negative, remaining tissue is cut at 100 μm to exhaustion. Metastases were always identified on H&E sections, but rapid immunohistochemistry for cytokeratins was useful as confirmation in 4%. This is time consuming (average 35 min and up to 65 min), but the definitive status of the SLN is available intraoperatively. The authors concede the disadvantages of this method with the volume of work involved, time required and the necessity for technician and pathologist to be available at the time of surgery.[31,32]

Molecular assessment

BACKGROUND

Histopathological examination of the SLN is essentially a sampling problem of identifying epithelial cells among large numbers of lymphoid cells. This is easily achieved using immunohistochemistry but can only examine a tiny percentage of the tissue in one section. An alternative approach has been to identify messenger RNA (mRNA) from genes expressed in epithelial cells but not in lymphoid cells.

Amplification of small amounts of mRNA using reverse transcriptase–polymerase chain reaction (RT-PCR) can be used to find epithelial-specific transcripts in lymph node homogenates. Numerous groups have applied this principle to the detection of metastatic breast cancer cells and melanoma in lymph nodes,[33–38] peripheral blood[39–41] and bone marrow.[42,43] In many cases the choice of probe for breast cancer cells was empirical, and a wide range of putative specific mRNAs such as carcinoembryonic antigen, CK19, CK20, GA733.2 and MUC1 were tested with variable results.[44] This research work has highlighted a number of problems with this approach. Early studies used a single probe, and it has emerged that sensitivity and specificity can be improved by employing multiple probes. Some studies did not use quantitative PCR but detected amplification products using Southern blot or ethidium bromide with simple positive or negative results. It became apparent that PCR can be too sensitive, leading to false-positive results, and that a better approach was to use quantitative PCR.[44–47] Setting of thresholds can exclude low-level mRNA expression perhaps from benign epithelial cells or tumour cells transported to nodes during palpation, needle biopsy or operation which are not clinically relevant.[48] There is some consensus that ITCs are not prognostically important and are regarded as N0 in the TNM classification, International Union Against Cancer, 6th edn.[11] The success of the molecular assays was judged in relation to the 'gold standard' provided by histological examination. The sensitivity of the histological 'gold standard' depends on the thoroughness of sampling and the use of immunohistochemistry. In some studies the lymph node was simply bisected and half submitted for molecular analysis and half for histology, which exacerbated the sampling problem.

When any PCR-based technique is compared with the histological assessment the findings are always as in Table 1. Concordant findings present no problem, but there are always cases in which cells are seen on H&E or immunohistochemistry but in which the test is negative.

Table 1. Possible outcomes and interpretations when a SLN is analysed for metastatic breast tumour cells using three methodologies: H&E, immunohistochemistry (IHC) and an assay based on molecular analysis (+, tumour present; –, tumour absent)

H&E	IHC	Molecular test	Interpretation
+	+	+	Concordance
+	+	–	?Molecular false negative ?Sampling
+	–	–	Probably sampling
–	–	–	Concordance
–	–	+	?Molecular false positive ?Sampling
–	+	+	Probably sampling

There is no way of knowing in these cases whether the test has failed producing a false-negative result or the finding is due to sampling error with no tumour present in the molecular test sample. Similarly, cases negative for histology will test positive and, once again, there is no knowing whether this is a false-positive test or a sampling problem. These problems emphasize the critical importance of rigorous controls to be included in every RT-PCR reaction to check the quality of the mRNA, exclude non-specific amplification and a positive control.

RECENT DEVELOPMENTS

More recently, further systematic searches have been performed for ideal markers of breast epithelial cells.[49,50] Analysis of gene expression in breast epithelial cells versus lymphoid cells has yielded two mRNA species that are most robust in distinguishing epithelial cells from lymphoid cells.[50] The mRNA protein products are CK19 and mammaglobin (MG). Specific PCR-based assays have been developed to identify mRNA for these two proteins in homogenates of lymph nodes. Previous application of PCR-based assays to detect metastatic disease has been a research activity requiring the availability of expertise and infrastructure to perform molecular techniques. The knowledge and experience acquired through more than a decade of research have been applied to the development of an assay that can be used in routine clinical practice. For the first time there is no requirement for specialist molecular laboratory and expertise. To minimize sampling problems, SLNs are cut into alternate slabs 2 mm thick and every other slab submitted for histological

examination with the intervening slabs assayed using the PCR method.

The commercial products available for clinical use contain reagents for lymph node homogenization, mRNA extraction, reverse transcription, amplification and fluorescence-based detection of amplicons. A thermocycling device to perform the cDNA amplification is about the size of a car battery and can be housed on a small trolley either in theatre or in the histopathology department. An internal positive control contains linearized plasmid cDNA for porphobilinogen, which is constitutively expressed in lymphoid tissue and acts to demonstrate that the amplification has proceeded. Once cDNA has been derived from the CK19 and MG mRNA, the PCR reaction produces cDNA complementary pairs that are known as amplicons. The amount of amplicon is detected using fluorphores with different excitation and emission wavelength for each molecule. The results are expressed as Ct values that are determined when the fluorescent signals reach a predefined level. The Ct value is a indication of the number of cycles required to reach the threshold value, hence, the smaller the amount of mRNA in the homogenate, the more cycles are required to reach the threshold and the Ct value is higher. The Ct value has been shown to be linearly related to the mRNA concentration (copies/ml) over an eight log range and the assay is estimated to be capable of detecting 100 mRNA molecules in the homogenate. If the Ct value of one or both of the epithelial markers exceeds preprogrammed limits and the controls are satisfactory, then software is used to provide a positive or negative result for clinical use. Trained biomedical science staff can perform the assay without supervision either in theatre or in the pathology department. The tissue homogenate must be assayed within 60 min or frozen at −65°C, but degrades beyond 21 days. mRNA extracted from fresh tissue can be frozen for 9 weeks, but no permanent archive of material is possible. Fat surrounding lymph nodes is inhibitory to the assay, and great care must be taken to avoid contamination of the specimens by breast tissue, breast tumour or amplicons from previous assays. Other possible contaminants, such as blood, blue dye and technetium, have no effect on the assay. In clinical trials the cut-off of the assay has been set at 2 mm. Overall agreement between the PCR-based assay and permanent section histology (which included examination of each lymph node at six levels with immunohistochemistry) was 92.7% (95% confidence interval 89.6, 95.1)[51] and for frozen sections at 50 μm intervals adjacent to the tissue submitted for RT-PCR 90.8% (sensitivity 77.8%, specificity 77.8%).[52]

USE OF A MOLECULAR ASSAY IN INTRAOPERATIVE ASSESSMENT

This assay replaces frozen section for detection of breast carcinoma in axillary lymph nodes. It reduces the sampling error associated with conventional 4 μm sections (either frozen or paraffin) and does not require the interpretation of a pathologist. It does, however, require the capital outlay, consumable costs and trained personnel, and there is no archival material available permanently.

The concordance with histopathological examination in multicentre trials of this assay has proven it to be effective.[51–53] Essentially, the histopathological read-out is semiquantitative (ITC, micrometastasis, macrometastasis) against semiquantitative read-out from the PCR assay. Based on the correlation between the histology and the PCR assay a cut-off (Ct value) can be chosen that roughly correlates with the histologically measured tumour burden. Since the clinically significant cut-off of metastatic burden, which should trigger axillary clearance on the one hand, and the offer of adjuvant chemotherapy on the other, is unknown, the cut-off in the PCR assay has no basis in scientific evidence. The classification of lymph node burden into ITC, Mi and macrometastasis on histological examination superficially appears sensible and straightforward, but in some cases classification is difficult with poor interobserver agreement. For example, when 20 pathologists were asked to classify the metastatic disease in Figure 1A, all classified it as Mi, but when classifying

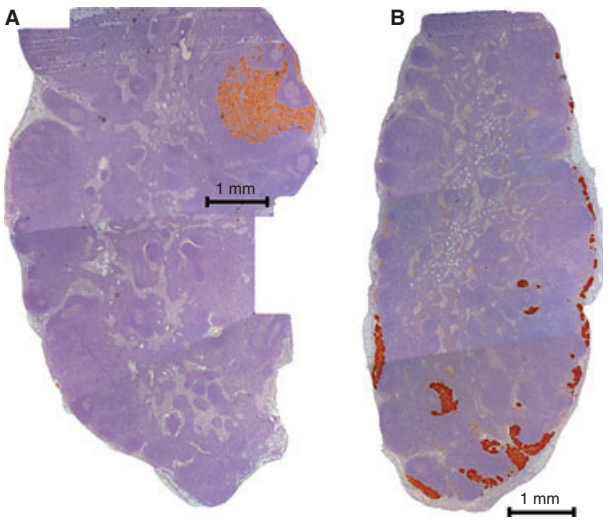

Figure 1. Photomontages of axillary lymph nodes (immunostained with AE1/AE3 for clarity at low power) sent to 20 pathologists who were asked to classify the metastatic disease present as macrometastasis, micrometastasis or ITC.

Figure 1B, half called it macrometastatic and half micrometastatic. Improvement can be achieved by more precise definition and description.[22,54] The quantitative data from PCR-based assays is an assessment of the total tumour burden in the node (the sum of the deposits in Figure 1B) and overcomes the difficulties of interobserver variation in interpretation illustrated in Figure 1. It seems self-evident that future clinical trials looking at the prognostic significance of small tumour burden in the sentinel node should include molecular data. The PCR assay has been approved by the Food and Drug Administration in the USA and by the European Union and is commercially available.

If this assay is used it raises a number of interesting issues. Should a sample of the lymph node be retained for formalin fixation, paraffin embedd and archiving, or should the whole node be submitted to the PCR assay to maximize sampling or minimize sampling error? The current recommendation is that the SLN(s) should be handled as in the validation studies, i.e. sliced fresh into 2 mm slabs and alternate slabs submitted for PCR and paraffin section histopathology. The node homogenates cannot be stored indefinitely, and this approach allows permanent archival material to be stored in paraffin wax. There are other arguments for retaining at least some material in paraffin.

It is important to recognize that the PCR assay cannot make other possible histological diagnoses in lymph node material (e.g. sarcoidosis, toxoplasmosis, mycobacterial infection, lymphoma). In some circumstances (after neoadjuvant chemotherapy with a complete response in the breast), the metastatic disease in the lymph node may be the only viable tumour remaining. If all nodal material is submitted for the assay, no tissue will be available for other clinically important assays (currently oestrogen receptor and Her2).

If the PCR assay is used intraoperatively and gives a positive result leading to an axillary clearance and no metastatic tumour is found on histological examination, how should the patient be managed? The problem is probably one of sampling, and the nodal metastasis must be small (at most a Mi <2 mm – the thickness of a tissue slab) and the Ct value is likely to be close to the cut-off between macro- and micrometastasis. The other axillary nodes are known to be histologically negative, so management as micrometastatic disease would be appropriate.

The Royal College of Pathologists recommendation is that paraffin blocks should be archived for 30 years. The mRNA extraction procedure results in a homogenate containing all the mRNA species in the tissue. Should this material be retained and stored? This material is known to have a limited life (9 weeks), but if storage is recommended there will be resource implications in providing appropriate deep freeze facilities.

If the use of the PCR assay reduces the sampling error associated with histological examination and more accurately reflects metastatic tumour burden in the axillary nodes, should all nodes in an axillary clearance be submitted and examined in this way? Currently, this would be more expensive than conventional histological examination (but costs may come down). More difficult is the problem that the axillary lymph nodes would need to be identified and dissected free of surrounding fat in the fresh state and assayed within 2 h of surgery. This would represent a significant change of practice and would almost certainly prove to be difficult and time consuming. Studies on the logistics and success of such an approach will be required to assess its cost effectiveness.

Conclusion

The development of CK- and MG-based assay for the detection of metastatic disease in breast cancer represents a significant translation from research into routine clinical practice. This technology can be readily adapted to other tumour types such as metastatic spread from prostatic cancer and melanoma.

References

1. Morton DL, Wen DR, Foshag LJ, Essner R, Cochran A. Intraoperative lymphatic mapping and selective cervical lymphadenectomy for early-stage melanomas of the head and neck. *J. Clin. Oncol.* 1993; **11;** 1751–1756.
2. Morton DL, Wen DR, Wong JH *et al.* Technical details of intraoperative lymphatic mapping for early stage melanoma. *Arch. Surg.* 1992; **127;** 392–399.
3. Cabanas RM. An approach for the treatment of penile carcinoma. *Cancer* 1977; **39;** 456–466.
4. den Bakker MA, van Weeszenberg A, de Kanter AY *et al.* Non-sentinel lymph node involvement in patients with breast cancer and sentinel node micrometastasis; too early to abandon axillary clearance. *J. Clin. Pathol.* 2002; **55;** 932–935.
5. Dixon M. Sentinel node biopsy in breast cancer. A promising technique, but it should not be introduced without proper trials. *BMJ (Clinical Research Ed)* 1998; **317;** 295–296.
6. Veronesi U, Paganelli G, Galimberti V *et al.* Sentinel-node biopsy to avoid axillary dissection in breast cancer with clinically negative lymph-nodes. *Lancet* 1997; **349;** 1864–1867.
7. Krag DN, Weaver DL, Alex JC, Fairbank JT. Surgical resection and radiolocalization of the sentinel lymph node in breast cancer using a gamma probe. *Surg. Oncol.* 1993; **2;** 335–339.
8. Kargozaran H, Shah M, Li Y *et al.* Concordance of peritumoral technetium 99m colloid and subareolar blue dye injection in breast cancer sentinel lymph node biopsy. *J. Surg. Res.* 2007; **143;** 126–129.

9. Turner RR, Ollila DW, Krasne DL, Giuliano AE. Histopathologic validation of the sentinel lymph node hypothesis for breast carcinoma. *Ann. Surg.* 1997; **226;** 271–276. discussion 276–278.
10. Mansfield L, Sosa I, Dionello R, Subramanian A, Devalia H, Mokbel K. Current management of the axilla in patients with clinically node-negative breast cancer: a nationwide survey of United Kingdom breast surgeons. *Int. Semin. Surg. Oncol.* 2007; **4;** 4.
11. Sobin LH. *UICC TNM classification of malignant tumours*, 6th edn. Chichester: John Wiley & Sons, 2002.
12. van Diest PJ. Histopathological workup of sentinel lymph nodes: how much is enough? *J. Clin. Pathol.* 1999; **52;** 871–873.
13. Greene FLPD, Fleming ID. *AJCC cancer staging handbook – TMN classification of malignant tumours*, 6th edn. New York: Springer, 2002.
14. Cserni G. Metastases in axillary sentinel lymph nodes in breast cancer as detected by intensive histopathological work up. *J. Clin. Pathol.* 1999; **52;** 922–924.
15. Cserni G, Amendoeira I, Apostolikas N *et al.* Discrepancies in current practice of pathological evaluation of sentinel lymph nodes in breast cancer. Results of a questionnaire based survey by the European Working Group for Breast Screening Pathology. *J. Clin. Pathol.* 2004; **57;** 695–701.
16. Cserni G, Gregori D, Merletti F *et al.* Meta-analysis of non-sentinel node metastases associated with micrometastatic sentinel nodes in breast cancer. *Br. J. Surg.* 2004; **91;** 1245–1252.
17. Viale G, Maiorano E, Pruneri G *et al.* Predicting the risk for additional axillary metastases in patients with breast carcinoma and positive sentinel lymph node biopsy. *Ann. Surg.* 2005; **241;** 319–325.
18. Weaver DL. Sentinel lymph nodes and breast carcinoma: which micrometastases are clinically significant? *Am. J. Surg. Pathol.* 2003; **27;** 842–845.
19. Klevesath MB, Bobrow LG, Pinder SE, Purushotham AD. The value of immunohistochemistry in sentinel lymph node histopathology in breast cancer. *Br. J. Cancer* 2005; **92;** 2201–2205.
20. Ellis IOPS, Bobrow L, Buley ID *et al. Pathology reporting of breast disease*. Sheffield: NHSBSP Publication No 58, 2005; 16–18.
21. Weaver DL. Pathological evaluation of sentinel lymph nodes in breast cancer: a practical academic perspective from America. *Histopathology* 2005; **46;** 702–706.
22. Cserni G. Evaluation of sentinel lymph nodes in breast cancer. *Histopathology* 2005; **46;** 697–702.
23. Lyman GH, Giuliano AE, Somerfield MR *et al.* American Society of Clinical Oncology guideline recommendations for sentinel lymph node biopsy in early-stage breast cancer. *J. Clin. Oncol.* 2005; **23;** 7703–7720.
24. Nathanson SD, Burke M, Slater R, Kapke A. Preoperative identification of the sentinel lymph node in breast cancer. *Ann. Surg. Oncol.* 2007; **14;** 3102–3110.
25. Memarsadeghi M, Riedl CC, Kaneider A *et al.* Axillary lymph node metastases in patients with breast carcinomas: assessment with nonenhanced versus uspio-enhanced MR imaging. *Radiology* 2006; **241;** 367–377.
26. Van Diest PJ, Torrenga H, Borgstein PJ *et al.* Reliability of intraoperative frozen section and imprint cytological investigation of sentinel lymph nodes in breast cancer. *Histopathology* 1999; **35;** 14–18.
27. Karsten U, Stosiek P. Fast and sensitive immunodetection of carcinoma cells in sentinel nodes. *Virchows Arch.* 2002; **440;** 325–329.
28. Salem AA, Douglas-Jones AG, Sweetland HM, Newcombe RG, Mansel RE. Evaluation of axillary lymph nodes using touch imprint cytology and immunohistochemistry. *British J. Surg.* 2002; **89;** 1386–1389.
29. Aihara T, Munakata S, Morino H, Takatsuka Y. Comparison of frozen section and touch imprint cytology for evaluation of sentinel lymph node metastasis in breast cancer. *Ann. Surg. Oncol.* 2004; **11;** 747–750.
30. Goyal A, Newcombe RG, Mansel RE. Clinical relevance of multiple sentinel nodes in patients with breast cancer. *British J. Surg.* 2005; **92;** 438–442.
31. Viale G, Bosari S, Mazzarol G *et al.* Intraoperative examination of axillary sentinel lymph nodes in breast carcinoma patients. *Cancer* 1999; **85;** 2433–2438.
32. Veronesi U, Paganelli G, Viale G *et al.* Sentinel lymph node biopsy and axillary dissection in breast cancer: results in a large series. *J. Natl. Cancer Instit.* 1999; **91;** 368–373.
33. Schoenfeld A, Luqmani Y, Smith D *et al.* Detection of breast cancer micrometastases in axillary lymph nodes by using polymerase chain reaction. *Cancer Res.* 1994; **54;** 2986–2990.
34. Noguchi S, Aihara T, Nakamori S *et al.* The detection of breast carcinoma micrometastases in axillary lymph nodes by means of reverse transcriptase-polymerase chain reaction. *Cancer* 1994; **74;** 1595–1600.
35. Mori M, Mimori K, Inoue H *et al.* Detection of cancer micrometastases in lymph nodes by reverse transcriptase-polymerase chain reaction. *Cancer Res.* 1995; **55;** 3417–3420.
36. Mori M, Mimori K, Ueo H *et al.* Clinical significance of molecular detection of carcinoma cells in lymph nodes and peripheral blood by reverse transcription-polymerase chain reaction in patients with gastrointestinal or breast carcinomas. *J. Clin. Oncol.* 1998; **16;** 128–132.
37. Liefers GJ, Cleton-Jansen AM, van de Velde CJ *et al.* Micrometastases and survival in stage II colorectal cancer. *N. Engl. J. Med.* 1998; **339;** 223–228.
38. Wlodzimierz R, Rutkowski P, Nowecki ZI, Kulik J, Nasierowska-Guttmejer A, Siedlecki JA. Detection of melanoma cells in the lymphatic drainage after lymph node dissection in melanoma patients by using two-marker reverse transcriptase-polymerase chain reaction assay. *Ann. Surg. Oncol.* 2004; **11;** 988–997.
39. Leitzel K, Lieu B, Curley E *et al.* Detection of cancer cells in peripheral blood of breast cancer patients using reverse transcription-polymerase chain reaction for epidermal growth factor receptor. *Clin. Cancer Res.* 1998; **4;** 3037–3043.
40. Zach O, Kasparu H, Krieger O, Hehenwarter W, Girschikofsky M, Lutz D. Detection of circulating mammary carcinoma cells in the peripheral blood of breast cancer patients via a nested reverse transcriptase polymerase chain reaction assay for mammaglobin mRNA. *J. Clin. Oncol.* 1999; **17;** 2015–2019.
41. Blaheta HJ, Paul T, Sotlar K *et al.* Detection of melanoma cells in sentinel lymph nodes, bone marrow and peripheral blood by a reverse transcription-polymerase chain reaction assay in patients with primary cutaneous melanoma: association with Breslow's tumour thickness. *Br. J. Dermatol.* 2001; **145;** 195–202.
42. Schoenfeld A, Kruger KH, Gomm J *et al.* The detection of micrometastases in the peripheral blood and bone marrow of patients with breast cancer using immunohistochemistry and reverse transcriptase polymerase chain reaction for keratin 19. *Eur. J. Cancer* 1997; **33;** 854–861.
43. Gerhard M, Juhl H, Kalthoff H, Schreiber HW, Wagener C, Neumaier M. Specific detection of carcinoembryonic antigen-expressing tumor cells in bone marrow aspirates by polymerase chain reaction. *J. Clin. Oncol.* 1994; **12;** 725–729.

44. Bostick PJ, Chatterjee S, Chi DD, Huynh TK, Giuliano AE, Cote R, Hoon DS. Limitations of specific reverse-transcriptase polymerase chain reaction markers in the detection of metastases in the lymph nodes and blood of breast cancer patients. *J. Clin. Oncol.* 1998; **16;** 2632–2640.
45. Zippelius A, Kufer P, Honold G *et al.* Limitations of reverse-transcriptase polymerase chain reaction analyses for detection of micrometastatic epithelial cancer cells in bone marrow. *J. Clin. Oncol.* 1997; **15;** 2701–2708.
46. Ko Y, Klinz M, Totzke G, Gouni-Berthold I, Sachinidis A, Vetter H. Limitations of the reverse transcription-polymerase chain reaction method for the detection of carcinoembryonic antigen-positive tumor cells in peripheral blood. *Clin. Cancer Res.* 1998; **4;** 2141–2146.
47. Inokuchi M, Ninomiya I, Tsugawa K, Terada I, Miwa K. Quantitative evaluation of metastases in axillary lymph nodes of breast cancer. *Br. J. Cancer* 2003; **89;** 1750–1756.
48. Carter BA, Jensen RA, Simpson JF, Page DL. Benign transport of breast epithelium into axillary lymph nodes after biopsy. *Am. J. Clin. Pathol.* 2000; **113;** 259–265.
49. Abdul-Rasool S, Kidson SH, Panieri E, Dent D, Pillay K, Hanekom GS. An evaluation of molecular markers for improved detection of breast cancer metastases in sentinel nodes. *J. Clin. Pathol.* 2006; **59;** 289–297.
50. Backus J, Laughlin T, Wang Y *et al.* Identification and characterization of optimal gene expression markers for detection of breast cancer metastasis. *J. Mol. Diagn.* 2005; **7;** 327–336.
51. Blumencranz P, Whitworth PW, Deck K *et al.* Scientific Impact Recognition Award. Sentinel node staging for breast cancer: intraoperative molecular pathology overcomes conventional histologic sampling errors. *Am. J. Surg.* 2007; **194;** 426–432.
52. Viale G, Dell'Orto P, Biasi MO *et al.* Comparative evaluation of an extensive histopathologic examination and a real-time reverse-transcription-polymerase chain reaction assay for mammaglobin and cytokeratin 19 on axillary sentinel lymph nodes of breast carcinoma patients. *Ann. Surg.* 2008; **247;** 136–142.
53. Mansel RE, Goyal A, Douglas-Jones AG *et al.* Clinical Trial: detection of breast cancer metastasis in sentinel lymph nodes using intra-operative real time GeneSearch TM BLN Assay in the operating room: results of the Cardiff study. *Breast Cancer Res. Treat.* 2008; (in press). [E Pub ahead of print. DOI:10.1007/s10549-008-0155-6.]
54. Turner RR, Weaver DL, Cserni G *et al.* Nodal stage classification for breast carcinoma: improving interobserver reproducibility through standardized histologic criteria and image-based training. *J. Clin. Oncol.* 2008; **26;** 258–263.

