What the clinician needs from the pathologist: evidence-based reporting in breast cancer

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Introduction

Histopathology is based in morphology, and this will continue for the foreseeable future. The National Institutes of Health (NIH) Consensus Conference on Adjuvant Therapy of Early Breast Cancer (November 1–3, 2000) agreed that no biochemical or molecular analysis of breast cancer apart from steroid receptor status had demonstrable application in therapeutic decision-making for individual patients. Once the age of the patient, tumour size, standardised pathological grade and/or mitotic rate, morphological tumour type, lymph node status and receptor status were taken into account, nothing else had any prognostic or predictive utility. Lack of quality control in the analysis and use of new candidate 'markers' has been a major problem.

Better understanding of carcinogenesis has revealed great complexity. Primary genetic changes influence morphology, but secondary and epigenetic changes lead to a complex genotype and phenotype. Carcinomas accumulate shared and unique abnormalities. The ability of the pathologist to 'compute' these changes optically, using decades of experience and proven clinical correlation justify the NIH statement. The challenge to the scientific community is to demonstrate that the genomic and proteomic revolutions have more to offer. Whether these techniques will supplement or even replace classical pathology as a diagnostic and predictive tool will depend on active collaboration between pathologists and the scientific community, with an emphasis on quality control and rigorous prospective study design.

This article examines the evidence base in pathology and highlights those parameters with proven utility in the management of patients with primary breast cancer. It does not deal with metastatic disease, except to indicate limitations to our current knowledge of the significance of micrometastases and circulating tumour cells.

Examination of the primary invasive carcinoma

Carcinoma size

For many years, it has been known that breast carcinoma size is an important independent prognostic variable [1–3], which the Nottingham Tenovus Primary Breast Cancer Study confirmed [4,5]. Tumour size is a key element of TNM (tumour, node, metastasis) staging, and the incidence of nodal metastases increases with tumour size [1].

Pathological measurement of carcinoma size is more accurate than clinical or radiological assessment, which may be altered by tissue changes not directly related to tumour infiltration. Carcinomas are best measured in the fresh specimen that should be submitted intact to the histopathology laboratory. This allows the histopathologist to assess the shape of the tumour, allowing best evaluation of tumour size and, ideally, to section the tumour in the plane of its largest dimensions. This also allows accurate assessment of the distance, grossly, between the edges of the tumour and resection margins of the specimen [6,7]. Carcinoma size should be verified histologically, whenever possible.

Breast Screening Programmes detect many invasive carcinomas smaller than 10 mm. In this situation, it is more appropriate to measure the size of the tumour microscopically using the Vernier scale or an eyepiece micrometer [9], as subdivision of tumours smaller than 2 cm (all T1 by TNM Staging [8]) into 5 mm bands allows further stratification of prognostic status and the likelihood of axillary nodal involvement.

Carcinoma grade

In 1925 Greenhough [9] recognised that the morphological appearance of the carcinoma and the degree of differentiation correlated with the degree of malignancy and eventual survival of the patient. Breast

practitioners worldwide are familiar with Bloom and Richardson grading, first published in 1957 [10] and subsequently adopted by the World Health Organisation, and the Nottingham modifications introduced in 1987 [11]. Histological grade in conjunction with stage improves the prediction of outcome [12], and grade may be particularly relevant to smaller, node-negative carcinomas. Grade is related directly to the probability of death from breast carcinoma, and inversely to the duration of disease-free and overall survival in lymph node-negative and node-positive individuals [12]. Different grading methods have been used throughout the world, with some groups preferring nuclear grading [13–15] and others favouring methods which introduce other morphological variables [10,11,16–18].

In the United Kingdom, the Nottingham modification of Bloom and Richardson grading has been adopted almost universally in response to clinical demand, and is an important component of the Nottingham Prognostic Index. The UK Breast Screening Programme requires registration of pathological grade as a Quality Assurance measure [19].

Older data suggesting that grade is subjective in character and poorly reproducible between pathologists [20,21] have contributed to a reluctance to accept grade as a reliable prognostic factor. However, the Nottingham group have shown that careful grading correctly allocates individuals to different prognostic groups [18], and that grading allows prognostic stratification in tumours from a multi-centre international study, confirming the results of previous Ludwig studies published in 1986 by Davis et al. [22].

Pathologists must adhere strictly to grading criteria. Guidelines and education programmes significantly improve grading consistency. To facilitate this, the UK Breast Screening Programme has published an illustrated guide [19] to ensure highly consistent and reproducible breast cancer grading between individual pathologists. In Europe, a similar approach is being pursued and all indications are that this will become policy in the US. The key, as recognised by NIH, is standardised grading. This requires the support of an educational external quality assurance (EQA) scheme. The additional prognostic information provided by grade was recently debated in *Cancer* [23–25].

Carcinoma type

Many carcinoma types are identifiable by light microscopy. While minor morphological variants may have little prognostic significance, certain special types of invasive breast carcinoma do imply more favourable outcomes. Tubular [26], cribriform [27], medullary [28,29], mucinous [30] and lobular carcinoma [31] all have improved survival compared to invasive ductal carcinoma of no special type (NST) [32].

Dixon and colleagues in Edinburgh have shown improved survival of individuals with tumours of special type, and that the incidence of these tumours is higher in the prevalent round of mammographic screening [33]. Among screening-detected carcinomas, the incidence of tubular carcinoma may be as high as 20%. This group have also demonstrated improved survival in lobular carcinomas compared to tumours of no special type. Among lobular carcinomas, survival is further stratified by subtype, with 60% 10 year survival for classical lobular, 90% for tubulo-lobular and 40% for the solid variant [31]. These data need to be confirmed.

The Nottingham group also have examined the relationship between tumour type and survival, and have suggested four broad prognostic groupings. An 'excellent' prognostic group includes tubular, cribriform, mucinous and tubulo-lobular carcinomas; a 'good' group of tubular mixed, mixed ductal and special type and classical lobular carcinomas; an 'average' group of mixed lobular, medullary and atypical medullary and a 'poor' prognostic group of ductal 'no special type' (NST), mixed ductal/lobular and solid lobular carcinoma [34].

Further studies from Nottingham have shown that allocation of patients to different prognostic groups using type and grade is more accurate than using histological type alone. The NIH consensus recognises the importance of tumour type.

Measurements of carcinoma cell proliferation

Techniques to estimate cell proliferation in routine practice need to be robust, reproducible, and simple. Counting mitoses in ten high-power fields of specified size achieves this. Counting mitoses per thousand tumour cells is theoretically attractive [35], but this and other markers of proliferation such as immunohistochemistry with the cell-cycle marker Ki-67, or flow cytometry to estimate S-phase fraction have not achieved routine acceptance. Mitotic counts form an essential component of tumour grading but mitotic counts on their own are also significantly prognostic [36]. Mitoses are hard to recognise reliably in poorly fixed tissue, so prompt and reliable transport of tissue to the pathology laboratory is vital.

Completeness of carcinoma excision

Evaluating the risk of residual carcinoma at the primary site requires careful histology of mastectomy and especially lumpectomy specimens. It is impossible to process for histology all the tissue submitted, but examining more blocks offers a greater chance of detecting carcinoma extending to a resection margin. The appropriate number of blocks is a compromise between exhaustive thoroughness and available resource. Histological sections are effectively two-dimensional, typically only 1/250th of a millimetre thick, so there is a further sampling problem in that even the tissue processed for histology cannot all be examined in a practicable number of sections. Observant selection of tissue for histology is required to maximise the chance of detecting incomplete excision. Inked resection margins are identifiable in histological sections, and orientation can be colour-coded. Closest margin should be identified at cut-up, confirmed microscopically, and recorded in millimetres for *in situ* and invasive carcinoma.

Breast cancer is often multifocal [37,38], so uninvolved resection margins do not guarantee complete excision. Diffuse infiltration, lobular histological type, or vascular invasion may increase the risk of residual satellite foci of cancer.

Ductal (DCIS) and lobular (LCIS) carcinoma *in situ* may extend widely within branching ducts. Microcalcification or stromal fibrosis can reveal their extent in X-rays or to gross examination [39], but may be absent and apparently clear resection margins are no guarantee that involved ducts do not reach a resection margin in a plane which has not been visualised histologically. There is no completely satisfactory solution to this problem in routine practice, but thorough histological sampling will show whether carcinoma *in situ* is localised or extensive, from which some estimate of the probability of residual disease can be made.

'Cavity shavings' and tumour bed biopsies are taken following wide local excision in some centres [40,41]. Negative histology reassures, but it has not been shown that such an approach is better than careful histological evaluation of a well-taken wide local excision. A possible advantage is that shavings can target an area of clinical concern identified during surgery. Clear cavity shavings do not guarantee complete excision of DCIS [42], and a 10 mm clear margin has been recommended for adequate surgical treatment of DCIS [42], a fairly stringent requirement.

Lymphovascular invasion

The distinction between invasion of blood vessels and lymphatic channels is not always morphologically obvious. Thus, all tumour emboli within endothelial-lined channels are often referred to as lymphovascular involvement or vascular invasion.

Many studies have identified lymphovascular invasion as a marker of poor prognosis [43–47], and tumour emboli in lymphovascular channels are associated with poorer overall survival [1–5] and local recurrence [48–50]. However, there have been many conflicting studies of the prognostic value of lymphovascular channel invasion. It has been suggested that this is due to a lack of consistency between histopathologists in the identification of lymphovascular spaces, but agreement between pathologists can be satisfactory [51]. Tissue spaces created by fixation and processing shrinkage artefact may be interpreted incorrectly as lymphatic channels [46]. This problem may be lessened by good fixation and processing, and searching for tumour emboli in spaces adjacent to the tumour rather than within its bulk [47]. Tumour emboli must be seen in a space lined by endothelial cells. These channels are usually within the breast stroma and are closely related to small blood vessels. Immunohistochemical staining with endothelial markers such as Factor VIII, CD34 and CD31 has not always proved reliable and is best used only in equivocal cases [52–54].

Lymphovascular channel involvement is a powerful predictor of local recurrence following breast-conserving cancer surgery or mastectomy [43–48] and is closely related to axillary nodal involvement [44,47], but lymphovascular tumour emboli are present in 5–10% of node-negative patients and are associated with earlier local recurrence and poorer overall survival [44,47,55–58].

Carcinoma *in situ* and microinvasive carcinoma

In carcinoma *in situ*, neoplastic epithelial cells line pre-existing breast ducts and lobules, anywhere from nipple epidermis (in Paget's disease) to breast periphery [59]. Extent varies greatly. Invasive and *in situ* carcinoma often occur together, consistent with the idea that invasive carcinoma often, if not always, develops from carcinoma *in situ*. Purely *in situ* carcinoma, without invasion, is more frequent in patients identified by mammographic screening than in symptomatic patients [60].

Despite their names, both 'ductal' and 'lobular' carcinoma *in situ* are defined by characteristic mor-

phologies, not by distribution. Indeed, molecular evidence suggests that LCIS and some low-grade DCIS are more closely related than low and high grade DCIS [61]. The distinction between atypical hyperplasia (ductal or lobular) and low-grade carcinoma *in situ* is somewhat arbitrary, and is not associated with a major discontinuity of subsequent invasive cancer risk.

As carcinoma *in situ* is not life-threatening *per se*, treatment is designed based on the perceived risk of invasive carcinoma. Descriptions of DCIS subtype (cribriform, solid, papillary) are of limited clinical significance: key determinants of recurrence risk appear to be nuclear grade and necrosis [62]. These are combined in the Van Nuys classification [63] and with extent and margin clearance in the Van Nuys prognostic index [64]. Ongoing studies compare competing prognostic classifications in DCIS [65,66], but in general, high-grade DCIS is appropriately treated as a malignant lesion requiring complete excision, and recurrences are frequently invasive.

Appraisal of complete excision in DCIS is, however, difficult and if extensive, mastectomy may be undertaken to achieve a high probability of cure. Appropriate surgery for low grade carcinoma *in situ* is less clear, even more so for the atypical hyperplasias and lobular carcinoma *in situ*, in both of which screening may be appropriate for a risk not necessarily localised to a particular area of the affected breast, and which may also apply to the other breast [67,68].

Microinvasive carcinoma is relatively unusual and implies minimally invasive disease foci (usually <1 mm) with carcinoma *in situ*. The probability of lymph node metastasis is low, but even without microinvasion, extensive high-grade DCIS is associated with occasional nodal metastasis. In these cases invasive carcinoma has been missed, so thorough histological sampling of DCIS is essential [69].

Evaluating the axilla in breast cancer

Detection (and removal) of axillary lymph nodes containing metastatic carcinoma is both prognostic and therapeutic. Women with node-negative disease get no therapeutic benefit from axillary clearance, but accurate staging is vital for correct treatment, because lymph node status remains one of the strongest prognostic factors in breast cancer, and because of this importance, level II axillary clearance is widely practised. The examining pathologist should recover every lymph node for histology. The number found depends on surgical and pathological dissection technique, and is sometimes used as a quality measure. A median of around 15 nodes or more can be expected, but varies widely [70], and occasionally fewer than ten nodes are recovered despite meticulous dissection. The number of positive nodes determines stage and derived indices such as the Nottingham Prognostic Index [71], so the total and number of positive nodes must be countable. Immunohistochemistry, molecular analysis and routine study of multiple histological levels may reveal otherwise undetected micrometastatic deposits, but as yet there is no evidence supporting routine use of such techniques.

Alternative approaches are four-node axillary sampling [72] and, more recently, sentinel node biopsy [73], which has been practised widely, but has yet to achieve universal acceptance, the possibility of understaging some patients being the chief obstacle.

The ability to identify micrometastatic deposits in lymph nodes or bone marrow has raised a large dilemma in clinical management, because relatively few studies have demonstrated relevance to outcome. The application of increasingly sophisticated techniques such as reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemistry to identify smaller numbers of tumour cells in nodes, distant tissue sites or blood has produced a further layer of confusion and complexity.

Significance of lymph node micrometastases

The classical publications of two decades ago indicated that lymph node metastases fewer than 2 mm detected by standard histological examination were of no proven importance for survival [74]. On the other hand, approximately 20% of node-negative patients will relapse with metastatic disease. This conundrum has led to more detailed analyses of lymph nodes, distant micrometastases and their significance.

Routine histopathological examination samples approximately 1% of the submitted tissue and has only a 1% chance of identifying a focus of cancer of 3 cell diameters [75,76]. Studies of serial sectioning of lymph nodes, with or without immunohistochemistry, show that false-negatives due to misreading of the initial slides are as frequent as increased detection from more detailed examination of the nodes [77,78]. Use of the term 'micrometastasis' for lesions less than 2 mm derives from the data that lesions of this size were of no clinical significance [74], but no pathologist would disregard nodal metastases approaching this size, and the term now encompasses tumour deposits down to single cells.

A definite survival disadvantage is associated with occult nodal micrometastases [79], but the size at

which they become significant has not been defined. Serial sectioning and immunohistochemistry increase the detection of nodal metastases by 9–33%, depending on the series [74], but serial sectioning with immunohistochemistry is not practical on all nodes in a routine laboratory. Large prospective studies are essential to establish the relative significance of micrometastases of different sizes, and accurate simulations [75,76] will guide the pathologist on the method of node sectioning and the number of sections to make certain of identifying lesions of specified size. Although lymph node examination remains the most important single prognostic factor in breast cancer, we still have not found the best way of identifying patients with involved nodes.

Hartveit originally suggested that deposits in the subcapsular sinus (embolic) may be associated with a worse prognosis [80]. It is interesting to note recent work from David Page's group indicating that carcinoma cell emboli in the subcapsular sinus may have been transported through the lymphatics to the nodes during surgery [81]. If so, the future behaviour of deposits in different parts of the node may have clinical significance, further complicating our present interpretations. As the number of nodes involved correlates with survival [80], other measures of tumour burden, such as the size of deposits will have a similar effect [7].

Continuing uncertainty on the significance of micrometastases of different sizes justifies pragmatism outside of clinical trials. Complete nodes too small to bisect easily (<3–4 mm) may be processed together in one cassette and should be examined in haematoxylin and eosin stained sections at 3–4 histological levels 250 μm apart, which in theory will give 100% accuracy of identifying micrometastases 250 μm in diameter [82]. All parts of any node cut through during dissection in the laboratory should be processed together in a cassette separate from other nodes. Nodes larger than 5 mm may be bisected through the plane of the hilum and both halves embedded or, better, sliced in a plane perpendicular to their longitudinal axis at 2–3 mm intervals and all slices processed together in one block. In this situation, one haematoxylin/eosin stained section per block is adequate. Reports must clearly specify the number of positive nodes and the total number of nodes examined.

It should be remembered that the only hard data from multicentre studies is based on 'routine examination of nodes', which is a variable interpretation.

Sentinel lymph node biopsy

With the introduction of breast screening programmes, tumours are smaller at detection and 80% of tumours are now lymph node-negative at the time of primary surgery, as assessed by routine procedures [83]. Formal axillary dissection is still considered the best staging procedure [84,85], but 80% of patients may therefore incur unnecessary morbidity. To overcome this, sentinel node examination was introduced. In the best hands, at least one sentinel node can be identified in more than 98% of patients and is 96% predictive of the axillary involvement [86,87]. Attempts to define molecular and biochemical signatures in the primary tumour that predict nodal involvement are without success to date [88]. If only a sentinel node is examined it is necessary to examine it thoroughly to ensure accurate prediction of the axillary status and to prevent false-negatives [86,87]. In some centres, it is feasible to carry out extensive intra-operative frozen section analysis [89] but in most centres limited frozen section reporting is available, and false-negative rates are high, particularly for small tumours [89] The NIH consensus concluded that there is insufficient evidence to justify adopting sentinel node as standard biopsy practice, and it should still be pursued within clinical trials [90].

Sentinel node biopsy was devised to reduce the requirement for axillary dissection, with its inherent morbidity, not as a procedure for detecting micrometastases. The extent to which small numbers of carcinoma cells in a sentinel node predict involvement of other axillary nodes, and their impact on prognosis, remains to be determined.

Bone marrow micrometastases

A 1998 meta-analysis [91] of 20 studies of bone marrow micrometastases in 2,494 patients concluded that "the prognostic impact of epithelial cells in the bone marrow remains to be substantiated by further studies using standardised methodic protocols". Most of the patients came from breast cancer cohorts. More recently the large Coombes study with a median 12.5 year follow-up of 350 patients concluded that immunohistochemical detection of micrometastases is associated with a reduced relapse-free and disease-free survival, but that this was not an independent prognostic variable when tumour size and nodal status were taken into account [92]. Clinical utility of other, more sensitive techniques of identifying tumour cells using PCR-based methods has yet to be demonstrated [93]. Detection of micrometas-

tases in the bone marrow has no proven clinical role at present and more high-quality clinical trials are required. It is essential that patient numbers are sufficient to give the statistical power to detect a likely predictive effect. This will require a large multicentre study and centralised quality control.

Circulating tumour cells

Circulating tumour cells in cancer have been known for approximately 150 years, but the use of RT-PCR to identify tumour cells and more recently immunomagnetic separation of tumour cells using antibodies have increased interest in this area. The main problems have been the lack of specificity of RT-PCR-based [94] and antibody-based [95] technologies. Cytogenetics with cell separation [96] may give increased specificity, but we are still defining the best methods to detect the cells, rather than being able to interpret their significance. An excellent review by Ghossein and Bhattacharya [97] makes depressing reading as it indicates the lack of scientific rigour with which most studies have been carried out.

New predictive and prognostic markers with unproven potential

HER2 (ERB-B2) and patient selection for adjuvant treatment

The cellular proto-oncogene *HER2* (also known as *ERB-B2* and *NEU*) is one of the most widely researched molecular prognostic factors in human breast cancer. Overexpression of its 185 kDa protein product is closely linked to gene amplification in breast, ovarian and stomach cancer [98–101]. The prognostic significance of *HER2* amplification/overexpression in breast cancer, first described by Slamon et al. in 1987 [98] has been comprehensively documented [102–108]. Despite evidence that *HER2* amplification/overexpression is independent of the classical carcinoma prognostic factors size, stage and grade [109], there remains controversy regarding node negative carcinomas [102,103,107,108], in which the small sample size of most of the studies hampers interpretation [110].

Although HER2 probably modulates responses to adjuvant chemotherapy and hormone therapy, there is insufficient evidence to warrant use of tumour *HER2* status in the selection of adjuvant therapies. Some studies suggest that *HER2* amplification is associated with tamoxifen resistance in vitro [111] and in vivo [112,113] but other studies find no such interaction [114,115]. Retrospective studies suggesting that *HER2* amplification/overexpression predicts cyclophosphamide, methotrexate, 5-fluorouracil (CMF) resistance [113,116–119] are contradicted by more recent evidence [13].

Three large clinical trials suggest that HER2 positive carcinomas are more sensitive to anthracycline-based chemotherapy [120–125]. Some smaller studies do not demonstrate this interaction, but nor do they suggest that HER2-positive carcinomas are anthracycline-resistant [110,126,127].

Most importantly, the novel agent Herceptin/Trastuzumab, which has significant anti-tumour activity as a monotherapy and in combination with chemotherapy in advanced breast cancer [120,128,129], will be the subject of clinical trials in adjuvant treatment in the immediate future. Testing for *HER2* status will be required in these clinical trials and perhaps eventually in a wider context [105,110,130].

This raises the question of how *HER2* status should be determined in clinical specimens. Controversy at a recent NCI conference on *HER2* testing over which assay should be used in clinical practice [105,110,130] focuses on the precision of immunohistochemistry (IHC) against more specialized molecular tests such as fluorescence *in situ* hybridization (FISH). Whilst other molecular tests are available (Southern blotting, PCR) [110], IHC and FISH remain the most accurate and widely available tests at present. Both tests have advantages and disadvantages, but at present there is too little evidence to recommend one over the other [131].

There is, however, some evidence from clinical trials that response to Herceptin is more accurately predicted by *HER2* amplification status than by p185 HER2 overexpression in fixed tissue [132]. Even patients with apparently strong overexpression by IHC failed to respond to Herceptin if no gene amplification was detected by FISH. Patients with moderate expression who are FISH-positive also have a high probability of responding to Herceptin. Confirmation in larger trials of Herceptin therapy will provide a strong argument for FISH in the routine diagnosis of *HER2* status.

Unlike most IHC tests, determination of HER2 status by IHC requires the scorer to determine the proportion of cells with positive staining and to categorise the intensity of staining. Evaluation of colour intensity is subjective and scorer bias well documented [133–135]. It is difficult to see how such bias can be avoided in multiple testing centres. In addition, intensity of IHC staining is dependent on tissue fixation conditions and the antibody used [136–139].

Such variation may be tolerable in 'all or nothing' tests, but not where signal quantitation is required [101,108,136–138]. In premenopausal women, menstrual cycle phase may also affect HER2 expression [140]. Finally, when compared with other measures of HER2 expression such as mRNA analysis or genuinely quantitative biochemical assays of p185 HER2 expression, FISH is probably a more accurate predictor of HER2 expression status [141] than IHC.

IHC should be used very cautiously for quantitation of markers in fixed tissues. Standardised fixation, antibodies, staining protocols and stringent EQA, perhaps with automated image analysis systems, *may* quantify expression, but this approach has never been rigorously evaluated. The 'Herceptest' relies on samples being fixed by a defined protocol, but unavoidable differences in fixation between samples may explain at least some variation in HER2 overexpression rates reported by centres using otherwise standardized tests [134]. Despite these challenges, available expertise in IHC and commitment to quality assurance (QA) in pathology laboratories in the UK and elsewhere represent an appropriate environment in which to solve the problems of *HER2* testing by IHC.

The study by Gusterson et al. [117] titrated the anti-HER2 antibody to detect only *HER2* amplified tumours, explaining the low percentage of tumours they detected expressing the protein. This logical approach could have saved much controversy in this area over the last five years.

FISH identifies patients likely to respond to Herceptin, and there is close correlation between HER2 overexpression and *HER2* amplification [103]. FISH predicts patient response more accurately even than 3+ IHC staining, and is especially helpful when IHC is equivocal [110,131]. Scoring FISH is more precise than scoring IHC, with interobserver error less than 10% readily achievable. However, FISH poses significant, although not insurmountable challenges if it is to replace IHC.

FISH is much used in haematopathology and prenatal diagnosis, but is only recently being applied to formalin-fixed solid tumour tissues with any frequency. Therefore the evidence base on the reproducibility, accuracy and precision of FISH in routine practice is relatively small. Criteria for scoring FISH are still evolving [131], and the importance of a centromeric control for FISH detection of *HER2* amplification has only recently been recognised [131,142]. Standard protocols will aid the evaluation of this novel technology. EQA is essential to the further development of FISH in tissue diagnostics [131]. Chromogenic *in situ* hybridisation may have application if permanent preparations are needed, and image capture may be used to archive FISH results.

Questions about the relative merits of FISH and IHC await resolution in appropriate clinical trials. Current evidence suggests that FISH testing for *HER2* status may be more accurate, reproducible and a better indicator of the potential response to Herceptin.

The challenge of future molecular predictive factors

This review has highlighted the diagnostic challenges of Herceptin, the first targeted molecular therapy in breast cancer. While there is not yet enough evidence to justify use of other candidate biological markers in breast cancer management, this may change. Many agents directed against molecular targets are in phase II and phase III clinical development. Most of these agents have specific molecular targets, identification of which will become an increasing part of the pathologists' remit. Agents targeting cell surface molecules, signal transduction pathways (protein kinase C, mitogen-activated protein kinase, phosphatidylinositol 3-kinase) or directed against mutated or otherwise modified proteins (p53) raise complex diagnostic issues. Agents targeting activated proteins (phosphorylated or ligand bound) will require diagnostic assays to identify the appropriate form of their target, for example phosphorylated epidermal growth factor receptor. Where gene alterations, mutation or duplication are the target, PCR or array-based mutation detection may be required (e.g. for TP53). As the field matures, combination therapies (for example targeting HER2 and its intracellular signalling pathways) may require complex panels of tests to 'map' molecular pathways in tumours.

Rigorous evaluation of novel tests to ensure accuracy, precision and clinical relevance will require stringent internal and EQA and prospective evaluation within carefully designed clinical trials.

Quality assurance

Twenty five years of experience of EQA for the measurement of oestrogen receptors (ER) [143] has demonstrated that EQA can dramatically improve inter-laboratory variation in measurement of a single parameter. However, it can only do this if in-house reproducibility is good. If absolute results for any single round of EQA are compared, even 'good' laboratories will differ from each other, but laboratories which report a high value for one particular standard will always be on the high side. Correspondingly,

'low' laboratories always report values below the mean. If a common external standard is circulated and the values from each laboratory are then normalised against the value obtained for this standard, values from the 'high' and the 'low' laboratories become comparable [143]. This suggests that EQA is essential for the reporting of any variable used in the treatment of any specific disease.

Accurate measurement of a particular parameter has to be necessary in the management of a disease to justify setting up an EQA system. ERs in breast cancer identify patients with a good chance of benefiting from endocrine therapy. Patients whose tumours are definitely receptor-negative will gain no anti-tumour benefit from endocrine therapy, and such treatment is a waste of resources. However, had endocrine therapy been the only treatment available, then measuring ER content would have been a waste of time.

Having established EQA for a particular parameter, other factors than just assay methodology can be relevant. Different methodologies may be needed for urine, serum or tissue [144]. Serum carcinoembryonic antigen (CEA) is valuable in monitoring colorectal cancer, but smokers may have higher circulating levels of CEA than non-smokers and serum CEA can be elevated in acute and chronic inflammatory conditions. CEA can be elevated in cancers other than colorectal. Different CEA kits give different values for a single common sample, so that the same test method must be used throughout any multi-centre study. A study of urokinase plasminogen activator [uPA] [145] showed that internal standards provided by different manufacturers could give different absolute values, confirming the need for EQA for any multi-centre study. Another key step [143,146] is to ensure that, when a particular laboratory fails to operate within the defined limits of the EQA scheme, remedial retraining procedures are established.

Even simple, well established procedures, such as determination of total protein content, can still be subject to significant variation in the best laboratories [143], demonstrating the need to check the quality of every step of each process including initial tissue handling and storage, through to sample preparation and even subsequent data analysis. As many laboratories switch increasingly to immunohistochemical methods for ER determination, it is critical [144] to ensure adequate, prompt fixation so that there is even penetration of the whole sample, use of a fully established antibody, a controlled and proven antigen retrieval system and a sensitive immunohistochemical detection method. Positive and negative controls must be included in each batch of staining. Where semi-quantitative analysis of immunohistochemical staining is required, QA of interpretation is just as important as that of methodology [147].

Oestrogen and progesterone receptor status

Duration of response of advanced breast cancer to endocrine therapy is proportional to the quantity of ER in the tumour [148]. The overview of early breast cancer treatment has shown also [149] that the benefit from adjuvant endocrine therapy is proportional to ER content. Finally, when tamoxifen is used for chemoprevention (or prevention of progression?) of breast cancer, there is a dramatic reduction in the number of ER+ carcinomas (treated versus controls), but no difference in the number of the ER− tumours between treated and control [150]. For all these reasons, there is a strong case for ER to be determined in all primary breast cancers. Immunohistochemical determination of ER is at least as powerful in predicting response to adjuvant therapy as biochemical measurement [151]. An appropriate methodology has been published [152] which recommends mandatory EQA and identifies schemes such as that run by UK NEQAS (contact rmkdhcr@ucl.ac.uk). Simple scoring is most effective, and a 'quick score' has been recommended [152]. This awards points for nuclear staining (0 = no nuclear staining; 1 = <1% nuclei staining; 2 = 1–10% nuclei staining; 3 = 11–33% staining; 4 = 34–66% staining; 5 = 67–100% staining) and up to 3 marks for intensity of stain (0 = no staining; 1 = weak staining; 2 = moderate staining; 3 = strong staining). If a tumour scores zero for ER using this system, the progesterone receptor (PR) content should be determined. Patients whose tumours are zero for both ER and PR will not respond to endocrine therapy and should receive alternate therapies, as appropriate. As the score for ER increases, so the chance of response to endocrine therapy increases.

The literature on ER status as a *prognostic* factor in its own right is difficult to interpret in the light of strong interactions with grade/differentiation and confounding in clinical trials. Undoubtedly, the importance of ER in clinical practice is its predictive value.

The detection of a second ER, now known as ER beta, makes matters a little more complex [153]. It is too early to comment on the relative amounts of the two types of receptor in breast cancer, or on the way that such a ratio might influence response to endocrine therapy.

Core data in breast cancer reporting

The following data are essential for logical decision-making in breast cancer treatment.

- Size of invasive carcinoma (maximum diameter), carcinoma grade, histological type.
- Minimum distance of invasive carcinoma to resection margin (surgical clearance).
- Presence or absence of lymphatic/blood vascular invasion.
- Extent and grade of associated carcinoma *in situ* and its surgical clearance.
- Number of axillary nodes containing metastatic carcinoma.
- Number of axillary nodes examined by histology.
- ER status (and ideally PR status if ER-negative).

Other data may have clinical utility but the data above represent a minimum standard which every laboratory should attempt to report in every case of invasive breast cancer.

Summary

Histopathology has a vital role in determining breast cancer management and pathologists must be part of the clinical team. Carcinoma size, grade, and especially lymph node status remain the best available prognostic factors. Metastatic carcinoma in axillary nodes is more important than any other prognostic factor presently available. ER status is an important predictor of response to endocrine manipulation, but its independent prognostic significance, and that of micrometastatic disease, circulating carcinoma cells and other molecular factors, even well-studied ones such as HER2 status, are less clear.

Pathology is the first clinical speciality to subject its practice to rigorous scientific analysis, and it has stood up well. However, workers without appropriate experience in Pathology or scientific design have created difficulties by undertaking poorly planned studies with ill-defined end-points, lacking appropriate quality control. New analytical techniques and therapeutic targets make it essential that we learn from past mistakes and integrate pathologists into the research teams pursing clinical trials and the assessment of new bio-markers. Without this, input resource will be wasted on false leads that could have been curtailed. Morphology alone will not be enough to select patients likely to benefit in trials of new therapies, but selection 'tests' must be appropriate. The confusion of tests for selection of patients to receive Herceptin shows what happens when this process fails. Much of the microarray data being put into data-bases has no quality control, and meta-analysis of this data will produce even more conflict than the clinical trials. This can be avoided, as the ability to standardise is available.

Abbreviations

CEA	Carcinoembryonic antigen
CMF	Cyclophosphamide, methotrexate, 5-fluorouracil
DCIS	Ductal carcinoma *in situ*
EQA	External quality assurance
ER	Oestrogen receptor
FISH	Fluorescence *in situ* hybridisation
HER2	p185 Her2 protein
HER2	*HER2* gene
IHC	Immunohistochemistry
ISH	*In situ* hybridisation
LCIS	Lobular carcinoma *in situ*
NEQAS	National External Quality Assurance Schemes
NIH	National Institutes of Health (USA)
NST	No special type
PCR	Polymerase chain reaction
PR	Progesterone receptor
QA	Quality assurance
RT-PCR	Reverse transcriptase PCR
TNM	Tumour, Nodal, Metastases staging system
UK	United Kingdom

Acknowledgements

Professor Gusterson's laboratory is supported by Breakthrough Breast Cancer.

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