Review

Measuring proliferation in breast cancer: practicalities and applications

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Abstract

Various methods are available for the measurement of proliferation rates in tumours, including mitotic counts, estimation of the fraction of cells in S-phase of the cell cycle and immunohistochemistry of proliferation-associated antigens. The evidence, advantages and disadvantages for each of these methods along with other novel approaches is reviewed in relation to breast cancer. The potential clinical applications of proliferative indices are discussed, including their use as prognostic indicators and predictors of response to systemic therapy.

Introduction

The development and continued growth of cancers involves altered rates of cell proliferation. In early breast cancer, measurement of proliferation can be used in conjunction with tumour size, grade, nodal status and steroid receptor status as a prognostic indicator [1,2]. Proliferation rates can provide useful information on prognosis and aggressiveness of individual cancers and can be used to guide treatment protocols in clinical practice. Adjuvant chemotherapy has been shown to improve survival in patients with breast cancer, but has potentially serious side effects. The potential of prognostic factors is to determine which patients are at higher risk of recurrence such that patients who stand to benefit more from adjuvant treatment can be identified. In the future, changes in proliferation rates during or after systemic therapy may be utilized as predictors of response and allow further tailoring of therapy. Information on proliferation rates is also necessary for the development of therapeutic agents, some of which may be targeted directly at specific points in the cell division pathway.

Various techniques have been developed to evaluate and quantify proliferation rates in the laboratory. Mitotic count estimates are widely used as a simple measure of cellular proliferation and are often incorporated into tumour grading systems [3]. Other methods have been developed, such as the detection of cells undergoing DNA synthesis using assays for thymidine uptake [4], flow cytometry to estimate the percentage of cells in S phase of the cell cycle or the detection of antigens associated with proliferation. This review will discuss current and developmental methods for assessing proliferation and the potential applications of such knowledge in the treatment of breast cancer. Table 1 summarises these methods and highlights their individual advantages and limitations.

Mitotic index

Cellular proliferation involves several defined phases. Cells in the resting (G0) phase are stimulated to enter the active cycle at the first gap (G1) phase. During this period of time, the cell prepares for DNA synthesis (the S phase), which is followed by a second phase of relative inactivity (G2) and preparation for the separation of the chromatids in the mitotic (M) phase. Cells can then recycle by entering the G1 phase or return to the resting G0 phase. Proliferation was first measured by counting mitotic bodies on paraffin-embedded tumour specimens stained using haematoxylin-eosin and viewed by microscopy. The characteristic appearance of the chromosome during M phase allows mitotic figures to be distinguished. The standard way of expressing the mitotic activity has been the number of mitotic bodies per high power field of view (HPF). A high mitotic count has been shown to be predictive of the risk of breast cancer death. Clayton [1] reported a study of 378 node-negative breast cancers and found that, on multivariate analysis, mitotic count was a stronger predictor of survival than tumour size, lymphatic

AgNOR = argyrophilic nucleolar organiser regions; BrdU = 5-bromodeoxyuridine; CDK = cyclin-dependent kinase; ER = oestrogen receptor; FDG = [^{18}F]-fluoro-2-deoxy-D-glucose; FLT = 3′-deoxy-3′-fluorothymidine; HPF = high power field of view; ^{3}HTdR = tritiated thymidine; LI = labelling index; PCNA = proliferating cell nuclear antigen; PET = positron emission tomography; SPF = S-phase fraction; TK = thymidine kinase; topoII = topoisomerase II.

Table 1

Methods of measuring proliferation

Method	Description	Advantages	Limitations
Mitotic index	Number of mitotic bodies on light microscopy	Cheap and simple staining method Can be used on paraffin-embedded specimens	Variability in counting Appearance of apoptosis/nuclear pyknosis can be confused with mitosis Relationship with proliferative rate might not be linear
S-phase fraction	Thymidine labelling index	Accurate even in slowly proliferating tumours Reproducible	Requires handling of radioisotope Requires time-consuming autoradiography Needs fresh tissue
	Flow cytometry	Can use on wide variety of tissue preparations Quick way of analysing many cells	Requires a relatively large tumour sample Poor reproducibility due to variability in tissue preparation and analysis between laboratories
	BrdU monoclonal antibodies/ immunohistochemistry	Better resolution and reproducibility than tritiated thymidine labelling No need for autoradiography	Requires fresh tissue and careful preparation Scoring can be time consuming
Nuclear antigen immunohistochemistry	Ki67/MIB-1 monoclonal antibody staining	Only need a small amount of tissue Sensitive Newer antibodies can be used on archival tissue	Scoring can be time consuming Variability in fixation can affect staining
	PCNA monoclonal antibody staining	Only need a small amount of tissue Sensitive	Poor correlations with other methods, prognostic factors and clinical outcome Scoring can be time consuming Variability in fixation can affect staining
Cyclins	Proteins that vary in expression during the cell cycle	Different cyclins associated with different cell cycle phases so can target cells committed to proliferation Can be performed on small, archival tissue samples Not influenced by stromal proliferation	Relatively new technique - not widely available for routine use
PET	Radiolabelled fluorothymidine incorporation detected by PET scans	Non-invasive Enables monitoring of proliferative changes during treatment Gives a global image of tumour, avoiding sampling errors due to heterogeneity	Patient exposure to radiation Yet to be verified as an accurate measure of proliferation Expensive and supply of radio-tracer is limited

BrdU, 5-bromodeoxyuridine; PCNA, proliferating cell nuclear antigen; PET, positron emission tomography.

invasion or skin invasion. Patients with more than 4.5 mitotic figures per 10 HPFs had a 2.8-fold increase in the risk of death. Various measures of tumour grade (nuclear grade, Bloom-Richardson grade, modified Scarff-Bloom-Richardson grade and Fisher's grade) were individually prognostic, but provided no additional predictive value when adjusted for mitotic count.

Variations in reported values for mitotic counts stem from the heterogeneity of tumour cellularity and from variations in the size of microscope HPFs. This can be circumvented to some extent by dividing the number of mitoses by the number of cancer cells in the field of view, although this makes the scoring process much more laborious. The scoring of mitotic index does seem to be relatively consistent in routine practice, as shown in a study by van Diest and colleagues [5]; 14 pathology laboratories scored 2,469 breast cancer specimens and the results were compared with those of a central laboratory. A mean correlation coefficient of 0.91 (range 0.81 to 0.96) was obtained. A prognostically relevant discrepancy was observed in 7.2% of cases (when the mitotic index scores would have resulted in different multivariate prognostic index estimates, based on mitotic index, tumour size and lymph node status). The reasons for the discrepancies were mainly due to poor tissue processing, inaccurate counting or failure to follow the guidelines for selection of the counting area [5].

One problem with this method is that it can be difficult to identify mitotic cells due to confusion with apoptosis or nuclear pyknosis. A further criticism of mitotic index as a measure of proliferation is that the duration of the mitotic phase of the cell cycle is variable, and hence the correlation of number of mitoses and proliferation rate is not necessarily linear [6].

S-phase

The measurement of the fraction of cells engaged in chromosomal DNA synthesis (the S-phase fraction (SPF)) has become one of the standard methods of assessing proliferation. The initial method of measuring SPF was by measuring the tritiated thymidine (^{3}HTdR) labelling index (LI) [7]. This method required the use of fresh material that was incubated with the DNA precursor, usually under high pressure oxygen conditions (to improve penetration to the tissue fragments) in an *in vitro* culture system. Autoradiography was then performed on the slides, usually several weeks after incorporation, and cells with overlying grains caused by the isotopic emission of ^{3}H were scored. The ^{3}HTdR LI tends to be much higher than the mitotic count because cells spend longer in S-phase than M-phase (approximately 7 to 24 hours as opposed to less than half an hour) [8]. This method allows accurate determination of proliferation rates even if proliferation is very slow, and does not have the problem of difficulty in identifying mitotic cells that is often encountered in mitotic index measurements. Meyer and colleagues [9] found the method to be reproducible with different observers scoring the radiographs and also found good correlation between primary tumours and their axillary metastases or recurrences. Correlation within and between laboratories is good, with coefficients of 0.96 and 0.93, respectively [10].

Tubiana and colleagues [2] measured the ^{3}HTdR LI in 128 breast cancer patients and compared this with survival after 10 years. Both relapse-free and overall survival were significantly higher in patients whose tumours had a low LI. As with the aforementioned work of Clayton using mitotic counts, the proliferation measure was more predictive of survival than other established prognostic factors such as tumour size, histological grade and number of involved lymph nodes. Similar results were confirmed by Meyer and colleagues, with ^{3}HTdR LI being predictive of overall survival on multivariate analysis along with nodal status, oestrogen receptor (ER) status and tumour size [11]. Measurement of ^{3}HTdR LI does, however, have limitations; it requires fresh tissue, it needs autoradiography, which is time consuming, and it requires the handling of a radioisotope.

Flow cytometry measurement of the SPF is perhaps the most clinically validated method for measuring proliferation. Its advantage is that it can be used on a wide variety of tissue preparations, including fresh surgical samples, frozen biopsy specimens and archival paraffin blocks [12]. However, there have been concerns over the standardization of both tissue preparation and analysis variability between laboratories. Cells are mechanically dispersed, stained with propidium iodide and passed through a flow cytometer, which produces a DNA histogram with distributions corresponding to phases in the cell cycle. The major peak corresponds to G1/G0 phase. DNA content (ploidy) and proliferation can be assessed. If there is a second major peak, the tumour is considered aneuploid, otherwise it is considered diploid. The SPF can be calculated using a simple algorithm. Flow cytometry measurements of SPF have been shown to correlate with mitotic counts, histological grades and ^{3}HTdR LIs [13,14]. Clark and colleagues [15] have demonstrated that measurements of ploidy and SPF using flow cytometry can predict disease-free and overall survival. A limitation of this technique is that samples contain varying contributions from normal stromal tissue and so the DNA histograms do not solely reflect the malignant component.

In 1982 Gratzner [16] described the use of monoclonal antibodies specific for 5-bromodeoxyuridine (BrdU) in the detection of DNA replication. This immunohistochemical method allows measurement of the SPF without the need for autoradiography or radioisotopes and has been shown to give similar results to ^{3}HTdR labelling [17]. Further work by Meyer and colleagues found BrdU labelling of breast cancer specimens to correlate with S-phase measured by ^{3}HTdR [18] and flow cytometry [14]. BrdU labelling has better resolution than ^{3}HTdR labelling, with less distortion of the nucleus or spill into the cytoplasm. Waldman and colleagues [19] found inter-observer reliability to be better for BrdU ($r = 0.94$) than ^{3}HTdR ($r = 0.87$) counting. Comparisons of BrdU counting and mitotic index have shown good correlations provided the mitotic figures are very carefully counted [20]. For successful labelling of S-phase cells *in vitro*, the tissue must be metabolically viable (that is, fresh) and adequately prepared (sliced less than 1 mm thick to allow the labelling agent to penetrate sufficiently). Also, thymidylate synthase should be blocked to enhance incorporation of the label rather than endogenous thymidylate.

Nuclear antigens

Rather than identifying cells engaged in particular phases of the growth cycle, an alternative method of assessing proliferation is to detect antigens that are closely associated with proliferation using immunohistochemistry. In theory these methods are quicker, cheaper and easier to use than flow cytometry and autoradiography and more reliable and reproducible than mitotic figure counting. They also have the advantage of being applicable to cytological material from breast aspirates and need less tissue than some other methods [21].

The Ki67 labelling index is now widely used as the measure of proliferation. Ki67 is a protein expressed in the nucleus during the cell cycle [22]. Cells express the antigen during G1, S, G2 and M phases, but not during G0. The original antibodies raised against Ki67 required fresh or frozen tumour specimens. Cells that showed specific nuclear staining were scored as positive and the Ki67 labelling index was expressed as the percentage of the total number of tumour cells that stain positive; this equates to the growth fraction of the tumour. Higher grade cancers have higher Ki67 indices - one study found mean scores of 9% in grade I tumours, 14% in grade II and 26% in grade III [23]. The Ki67

index correlates significantly with estimates of the mitotic index [24] and SPF by flow cytometry [13,25,26], although some of the published correlation coefficients are modest ($r = 0.42$ [13], $r = 0.22$ [26]). Various studies have shown correlations between Ki67 and disease-free and overall survival, with an increased risk of recurrence in tumours with a high Ki67 [27-33]. Pierga and colleagues [34] performed a multivariate analysis and showed the Ki67-determined growth fraction to be an independent prognostic factor ($p = 0.03$) along with nodal status, age and adjuvant treatment received.

Newer antibodies such as MIB-1 identify peptides from recombinant fragments of the gene for the Ki67 antigen, and have the advantage of being effective in fixed, archival specimens following microwave irradiation. Staining with the MIB-1 monoclonal antibody has been shown to correlate well with histological grade, mitotic index, relapse-free interval and overall survival [35,36]. Attempts have been made to define cut-off values to classify tumours as having high or low proliferative activity. Spyratos and colleagues [35] performed multivariate analysis on 185 breast tumours using 5 different cut-off values of MIB-1 staining. A MIB-1 cut-off of 25% was best for correctly identifying highly proliferative tumours (although to confidently identify tumours with low proliferative potential, a cut-off of less than 10% was required).

Other nuclear antigens, such as proliferating cell nuclear antigen (PCNA), have been investigated, but appear to correlate poorly with Ki67 and mitotic count so may be of more limited use in assessing proliferation [23]. Unlike Ki67, PCNA is also involved in DNA repair processes, which may be a confounding factor in cancer. Clark and colleagues [37] reported on a pilot study using a monoclonal antibody against mitosin (a recently described 350 kDa nuclear phosphoprotein that is expressed in the late G1, S, G2, and M phases of the cell cycle but not in G0), finding its expression to correlate strongly with SPF in a series of 386 formalin-fixed archival breast cancers. Although there was no relation with overall survival in this study, they did observe that a high mitosin level significantly correlated with recurrence in multivariate analysis (SPF did not correlate with recurrence) [37].

Cyclins and cyclin-dependent kinases

Progression through various stages of the cell cycle is dependent on the presence of complexes formed between cyclins and cyclin-dependent kinases (CDKs). Cyclins are proteins that vary in expression during different phases of the cell cycle. Cyclin D1 is expressed during G1 phase, cyclin E during G1 and early S phase, cyclin A during S and G2 phase and cyclin B during late G2 phase [38-41]. They are, therefore, useful markers of the proportion of cells in given phases of the cell cycle at any one time. Moreover, various cyclins, such as cyclins D1 and E, have been shown to be elevated in malignancy [42-44]. High expression of cyclins A [45,46] and E [47-50] is associated with a poor prognosis in breast cancer. The evidence for a prognostic role of cyclin D1 is less convincing but overexpression appears to be linked to hormone receptor-positivity and there is some evidence of a relationship between high levels of expression and a good prognosis [51-53].

As with Ki67, staining for cyclins is achieved using immunohistochemistry with specific monoclonal antibodies. It can thus be performed on paraffin-embedded material and tumour-specific expression is discriminated from stromal staining by morphology. Correlations between expression of various cyclins and Ki67 measurements of proliferation have been demonstrated [43,45-47]. However, cyclins have an advantage in that they can selectively detect for cells that are in the late G1 phase and beyond and thus committed to cell division. This property might have a clinical benefit when considering using cytotoxic agents that target specific points in the cell cycle [54].

Inhibitors of the CDKs can also be studied using immunohistochemical techniques. p27 is one such inhibitory protein; it binds to and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes, and thus controls the cell cycle progression at G1. Low nuclear p27 levels (and sequestration of p27 in the cytoplasm) are associated with high proliferative activity and have been shown to relate to a high tumour grade and poor prognosis [55-59]. Interestingly, there appears to be a significant correlation between low p27 expression and overexpression of HER2/neu in breast tumours, and it has been suggested that the HER2/neu product might have a role in down-regulating p27 expression [60,61].

Another protein to interact with CDK complexes is p21 (WAF1/CIP1). p21 binds to and inhibits the activity of cyclin-CDK2 or -CDK4 complexes, and thus also functions as a regulator of cell cycle progression at G1. p21 expression is tightly controlled by the tumour suppressor protein p53, through which it mediates the p53-dependent cell cycle G1 phase arrest in response to a variety of stress stimuli. p21 also interacts with PCNA and is involved in the regulation of S phase DNA replication and DNA damage repair. The prognostic value of p21 is under debate, with some studies showing low expression to be a favourable marker in node negative patients, and others showing no prognostic value [62-65].

Other methods

Argyrophilic nucleolar organiser regions (AgNORs) are non-histone proteins associated with loops of DNA actively transcribing to ribosomal RNA. The number and size of AgNORs can be assessed following staining of the tumour tissue with silver stains. During the mitotic cycle there is aggregation and segregation of NORs. Immediately after mitosis the NORs are dispersed through the nucleus and the nucleolus is not readily apparent. AgNOR staining would reveal a large number of dots. The NORs then cluster to form one or more nucleoli and AgNOR staining then reveals fewer

dots because the NORs have coalesced. In late G2, the NORs tend to disperse with dissolution of the nucleolus. The most extreme segregation of NORs is seen during mitosis when the chromosomes separate. Thus, the AgNOR count may be higher in cells in late G2 or early G1 when the NORs are segregated and they are more easily discernable.

The number of AgNORs is increased in malignancy, but is not diagnostic due to overlap with benign proliferation [66]. It seems that, although the number of AgNORs per cell is not discriminatory enough on its own to determine malignancy, the addition of size or area measurements using image analysis gives improved diagnostic and prognostic specificity [67,68]. AgNOR counts can be obtained successfully from fine-needle aspiration smears [69]. The AgNOR score has shown a positive correlation with DNA ploidy, tumour grade and the SPF on flow cytometry [70-72], with Ki67 staining [73-76] and with PCNA [77] and has, therefore, been proposed as an alternative measure of tumour proliferation. As with the aforementioned immunohistochemical techniques, the scoring can be time consuming and there may be problems with reproducibility [74].

The enzyme thymidine kinase (TK)1 is involved in the phosphorylation of deoxythymidine during DNA synthesis. It is present in the cytoplasm and activated at the late G1 phase of the cell cycle. TK1 activity is high in proliferating and malignant cells, but is low or absent in quiescent cells. Wang and colleagues [78] have developed a polyclonal anti-TK1 antibody and demonstrated cell cycle-dependent expression of the enzyme. There is a good correlation with MIB-1 antibody staining [35] and the antibody can be used on archival tissue. Romain and colleagues [79] studied 154 node-positive breast cancers treated with chemotherapy and found that patients whose tumours had higher levels of TK1 activity had an increased risk of relapse or death.

The nuclear enzyme topoisomerase II (topoII) breaks and rejoins strands of DNA. The isoform topoIIα is a marker of cell proliferation and is also the molecular target for the anthracycline class of chemotherapy drugs commonly used in the treatment of breast cancer. Various studies have shown that tumours with higher baseline levels of topoIIα tend to be more responsive to anthracycline chemotherapy [80-82] whilst others have found that high baseline levels are a poor prognostic factor, predicting for poor five-year disease-free survival [83]. Jarvinen and colleagues [84] evaluated topoIIα expression immunohistochemically in 230 breast cancer specimens and found a highly significant correlation with tumour proliferation rate measured by SPF ($p < 0.0001$). This association with proliferation has been confirmed by other investigators using MIB-1/Ki67 expression [85-87], and the general consensus is that increased topoIIα expression gives information on the number of cycling tumour cells and is linked with an aggressive tumour phenotype.

Recently, Misell [88] and colleagues reported on a new method of measuring proliferation *in vivo* using heavy water labelling followed by mass spectrometry analysis. Women were given daily doses of heavy water for one to four weeks prior to mastectomy (or biopsy in healthy volunteers) and significantly higher proliferation rates were seen in pre-menopausal than in post-menopausal women, with different proliferative patterns in tumour cells. The authors claim that this method might be more reproducible than the immunohistochemical scoring methods, particularly in cases with relatively low proliferation rates.

Recent developments in tissue microarray technology have enabled the analysis of multiple targets at the DNA, RNA or protein level on sections containing hundreds of tumour samples. High-throughput tissue microarrays can be used to screen for genes with differential expression between cancer cells and normal tissue [89,90] and gene expression signatures have been developed that can predict survival in breast cancer [91,92]. Dai and colleagues [93] found that the occurrence of metastases in breast cancer could be predicted by a homogeneous gene expression pattern consisting almost entirely of cell cycle genes. Overexpression of this set of genes is related to an extremely poor outcome in a subset of patients with strong ER expression. Overexpression of cell cycle genes is indicative of cell proliferation, so microarray technology provides an alternative to proliferation assays. However, before microarrays are used routinely in assessing proliferative activity of individual tumours, there needs to be improvements in both cost and logistics.

Positron emission tomography

Thymidine has shown some potential as a tracer for use in positron emission tomography (PET) scanning and early studies in humans have shown correlation of 2-[^{11}C]-thymidine uptake with tumour activity [94]. However, the short half-life (20 minutes) of [^{11}C] and the catabolism of thymidine made the tracer impractical for routine clinical use. 3′-Deoxy-3′-fluorothymidine (FLT) is an analogue of thymidine that was initially developed for the treatment of HIV, but was found to cause myelosuppression, peripheral neuropathy and nausea at therapeutic doses. However, when used in tracer doses with an [^{18}F] label it is both non-toxic and has the advantage of a longer half-life (110 minutes) than 2-[^{11}C]-thymidine. The use of FLT in PET scanning was introduced by Shields and colleagues [95]. Thymidine is rapidly transported into the cell from the extracellular fluid using non-energy-dependent nucleoside transporters and active, Na^+-dependent carriers. After entering the cell, FLT is converted to a monophosphate by the enzyme TK1. The monophosphate lacks a hydroxyl group, thus preventing its incorporation into DNA and trapping it within the cell. As mentioned above, the activity of the TK1 enzyme increases dramatically during DNA synthesis. The uptake of FLT is related to TK1 activity, and so is linked to proliferation. The development of a non-invasive measure of proliferation that does not require biopsy

specimens may allow the future monitoring of changes in proliferation of tumours in patients undergoing treatment.

A good correlation between standardised uptake value measures of FLT uptake and Ki67 proliferation marker scores has been demonstrated in a variety of human cancers, including non-small cell lung cancer, lymphoma, colorectal cancer, soft tissue sarcoma and breast cancer [96]. Ten patients with suspected or proven non-small cell lung cancer underwent FLT-PET and Ki67 staining was performed on tissue specimens. Strong correlations were seen between Ki67 scores and four different definitions of FLT uptake (average, partial-volume-corrected and maximum standardised uptake values and average FLT flux). In breast cancer, a pilot study showed a highly significant correlation ($r = 0.76$-0.94) between Ki-67 LI and a variety of different kinetic parameters of [^{18}F]FLT retention in 12 evaluable patients with breast cancer [97]. Comparisons with the standard PET tracer, [^{18}F]-fluoro-2-deoxy-D-glucose (FDG), a measure of metabolism rather than proliferation, have shown FLT to be more specific for tumour activity [98,99]. However, FLT is not a substitute for FDG for tumour staging due to its lower uptake (approximately 50% of FDG levels). FLT has other limitations, including a high background uptake in the liver [100], which precludes the imaging of liver tumours, and there are concerns about radiation exposure if patients are to undergo multiple scans, particularly in women receiving curative treatment. However, studies are underway to assess the usefulness of FLT in predicting the response of breast cancer to chemotherapy [101,102].

Discussion

There are two important potential applications of proliferation measurement in clinical practice. Firstly, there might be a role in predicting prognosis, thus improving the physician's ability to identify the patients most likely to benefit from systemic adjuvant therapy. As previously discussed, each of the major methods of measuring proliferation has revealed evidence of correlations of proliferative rate with recurrence and overall survival [1,2,11,15,27-29]. This information could be particularly important in patients in whom adjuvant therapies might not be recommended on the basis of staging information alone, such as those with small, node negative tumours. Some studies show the prognostic value of proliferation index to be more significant in patients with T1 and/or node negative tumours [34,36]. In these patients, if the proliferative rate is high, adjuvant treatment might be considered where it might not be otherwise. Tumour grade is already used in clinical practice when determining recommendations for adjuvant therapy, and this is partly dependent upon mitotic count and hence proliferation. The more specific measures of proliferation discussed above are not routinely used to influence this decision, but as their use becomes more widespread it would seem reasonable to add them into future protocols for study alongside other established prognostic factors. Table 2 summarizes the evidence for using measures of proliferation when determining prognosis.

The second potential application of proliferation measurement in clinical practice is in predicting response to treatment. The use of primary systemic therapy for the treatment of tumours that have not yet been resected allows the opportunity to assess the response during a course of therapy. Attempts have been made to identify baseline markers that can predict for a subsequent response and thus allow tailoring of treatment to best suit an individual [103]. Perhaps what is of more interest is the ability to observe changes in these markers over a course of treatment. Of the various parameters tested (including ER, progesterone and HER-2 receptors, bcl-2, ploidy, p53, Ki67 and SPF), changes in proliferation appear to be most promising. Makris, Chang and colleagues [104] have shown that changes in Ki67 index, two to three weeks after commencing tamoxifen and chemotherapy (mitoxantrone, methotrexate with or without mitomycin), are predictive of response. These studies examined a variety of cytological markers before treatment and repeated ten days to three weeks after commencing treatment and compared these with clinical response after four cycles of chemotherapy. A decrease in Ki67 score at three weeks significantly predicted for subsequent good clinical response. More recently, Burcombe, Makris and colleagues [105] studied 118 breast cancer patients treated with 6 cycles of neoadjuvant anthracycline-based chemotherapy. Diagnostic biopsies and post-chemotherapy surgical specimens were stained for ER, progesterone receptor, HER-2 and Ki67. No single pre-treatment parameter predicted for response, but tumours displaying larger reductions in Ki67 after treatment were more likely to have achieved a pathological response. Similar results have been confirmed with the aromatase inhibitor anastrozole. The IMPACT study compared 12 weeks of neoadjuvant treatment with anastrozole, tamoxifen or the combination of both drugs in 330 post-menopausal women [106]. Although clinical response measurements and final surgical outcomes were no different between the groups, neoadjuvant anastrozole resulted in a greater reduction in Ki67 scoring after two weeks than either tamoxifen or the combination [107]. It is suggested that this might parallel the emerging evidence that adjuvant aromatase inhibitors achieve a greater reduction in relapse rates of breast cancer when compared with tamoxifen or the combination [108].

It is important to note that, although a decrease in Ki67 score may predict well for patients who will subsequently respond to treatment, it appears that the absence of a significant change in Ki67 does not mean that the patient will not respond. Assersohn and colleagues [109] looked at changes in Ki67 values in patients treated with chemotherapy and hormone therapy and compared these changes with response to treatment. Positive predictive values for response were 85%, but negative predictive values were poor at 59%. It would, therefore, be hard to justify a change in treatment of

Table 2

Clinical applications of measures of proliferation in breast cancer

Application	Evidence	Reference
Prognostic indicator	High mitotic count predictive of risk of breast cancer death (relative risk = 2.8)	[1]
	High thymidine labelling index correlates with worse relapse-free and overall survival (significance differs by subgroups, p = 0.16 to 0.0002)	[2,11]
	Measures of S-phase fraction and DNA ploidy by flow cytometry can predict for disease-free and overall survival (p = 0.007)	[12]
	On multivariate analysis Ki67 score is independently predictive of disease-free survival (p = 0.038) and relapse free survival (p = 0.03)	[29,34]
	High expression of cyclins A and E associated with poor prognosis	[45-50]
Planning adjuvant treatment	In some studies the prognostic value of proliferation index is particularly significant in patients with T1 and/or node negative tumours (in whom chemotherapy might not otherwise be advised)	[34,36]
Prediction of response	Changes in Ki67 after one cycle of chemotherapy predict eventual clinical response (p = 0.05)	[104]
	Changes in FLT-PET uptake after one cycle of chemotherapy predict eventual response on CT-imaging (r = 0.79)	[101]

FLT, 3′-deoxy-3′-fluorothymidine; PET, positron emission tomography.

an individual patient on the basis of lack of Ki67 reduction alone.

Two randomised phase III studies have addressed the role of adjuvant chemotherapy on the basis of tumour proliferation measurements. Paradiso and colleagues [110] used ^{3}H-thymidine autoradiography to determine proliferative activity in the tumours of women with node negative breast cancer. Those with a high LI (>2.3%) were randomised to receive adjuvant anthracycline-based chemotherapy versus no adjuvant therapy. Five-year disease-free survival was 81% in the chemotherapy group versus 69% in the control arm ($p < 0.02$), suggesting that proliferation measurement might help identify patients who would benefit from chemotherapy. Similarly, in the peri-operative setting, Pronzato and colleagues [111] found a significant improvement in survival in node-negative patients with a high thymidine LI who underwent chemotherapy.

It has been suggested that it is simplistic to view cells in the context of either cycling or non-cycling. The non-cycling compartment is heterogeneous, containing non-reproductive end-stage cells and reproductive cells that are dormant. Baker and colleagues [112] describe an *in vitro* analysis that can differentiate between these components. They define a new parameter, the cycling reproductive fraction, which is the fraction of all cells with reproductive capacity that are currently active in the cell cycle. In some tumours the cycling reproductive fraction can approach 100%, but in others can be much lower. Perhaps by taking this into account in future studies, the prognostic and predictive value of proliferation can be improved.

It is somewhat impractical for a patient to have an additional biopsy two to three weeks into their treatment and studies are now ongoing into the utility of FLT-PET imaging in the prediction of response to chemotherapy. There has been some early promise shown in studies using FDG-PET (a measure of metabolism rather than proliferation). Patients were imaged with FDG-PET prior to neoadjuvant chemotherapy and again after one or two cycles. Changes in FDG uptake between the two scans were correlated with eventual pathological response determined at surgery on completion of the course of chemotherapy. Tumours that went on to pathological response were found to have a sharp decrease in FDG uptake (often down to background level), whilst non-responding lesions showed little change in tracer uptake. After the first course of chemotherapy, all responders could be identified by a decrease in standardised uptake values below 55% of the baseline (sensitivity 100%, specificity 85%). This resulted in an accuracy of 88% in predicting histological response after one cycle of therapy (91% after two cycles) [113]. It remains to be seen whether similar results can be achieved using the more specific marker of proliferation, FLT. Pio and colleagues [101] have demonstrated that changes in FLT uptake during treatment for breast cancer correlate with changes in serum tumour markers and the tumour size on imaging.

Conclusion

Various methods have been validated as measures of proliferation, including mitotic body counting, immunohistochemical staining of antigens associated with proliferation or the estimation of the fraction of cells in S-phase by flow cytometry or the incorporation of thymidine or BrdU. Each of

these methods has been shown to have prognostic value in breast cancer, but all require biopsy or surgical samples of tumour tissue. This does lead to several limitations: biopsies are invasive and involve a degree of patient discomfort; deep-seated tumours may not be amenable to biopsy; the biopsy may not be representative of the whole tumour, as tumour heterogeneity is well described; and the scoring methods are partly subjective and, therefore, variable.

For these reasons the development of non-invasive, reproducible and validated methods of proliferation measurement will be a major advance for the evaluation of anti-neoplastic agents and for identifying non-responders early in their treatment so that they can be offered alternative and possibly more efficacious therapies.

The use of functional imaging techniques such as FLT-PET may overcome some of these barriers. Future studies should correlate immunohistochemistry and functional imaging estimates of proliferative activity at baseline and repeated early in the course of treatment, and compare both with response outcomes. In the meantime, if proliferation is to be used as a prognostic or predictive factor, it is important for pathology reports to use a standardized technique. Until the reliability of these new methods is confirmed, the current standard proliferation assay should be Ki67 immunohistochemistry, given its relative simplicity and wide availability.

Competing interests

The authors declare that they have no competing interests.

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