Proliferation Markers in Breast Cancer

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Abstract

Breast cancer proliferation can be measured by several approaches (mitotic count, S-phase fraction assessment, evaluation of cell cycle-related protein expression, molecular tests). It is used to predict outcome, by discriminating luminal A from luminal B breast cancer, and consequently to guide the choice of chemotherapy in hormone receptor-positive, HER2-negative breast cancer. Proliferation reflects tumor aggressiveness and gives valuable information for the identification of patients at risk of early relapses and thus potentially candidates for chemotherapy. Dynamic evaluation of proliferation allows identification of the patients resistant to neoadjuvant endocrine treatment and, at a lesser degree, to neoadjuvant chemotherapy. Evaluation of proliferation does not bring any added value to the management of HER2-positive, triple negative or metastatic breast cancer.

Keywords

Breast · Cancer · Proliferation · S-phase · Immunohistochemistry · Gene expression · Prognosis

7.1 Introduction

Uncontrolled proliferation is one of the hallmarks of cancer as proposed by Hanahan and Weinberg (2011), so numerous studies have been published about proliferation activity of breast cancer (BC). Before the era of gene expression profiling, proliferation was evaluated by counting mitoses, by flow cytometry to determine the S-phase fraction or by immunohistochemistry (IHC) to assess the expression of proliferating cell nuclear antigen (PCNA), Ki67 or the related proteins.

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Breast cancer is a heterogeneous disease involving at least four relevant molecular subgroups (two oestrogen receptor (ER)-positive: luminal A and B, and two ER-negative: HER2-enriched and basal-like) (Lakhani et al. 2012; Perou et al. 2000). Those molecular subgroups, known also as breast cancer intrinsic subtypes, are defined by gene expression profiles. They harbour distinct clinical features including prognosis and metastatic behaviour. Luminal breast cancers can be further divided into luminal A and luminal B subtypes, mainly upon the expression level of proliferation genes, such as *MKI67*, *AURKA*, *TOP2A*. Luminal B cancers have higher proliferation rates and poorer prognosis than luminal A cancers. This is the reason why expression of Ki67 (Gerdes et al. 1983) has been used to distinguish immunohistochemically defined luminal A from luminal B breast cancers in the 2013 and 2015 Saint Gallen consensus conference (Goldhirsch et al. 2013; Coates et al. 2015).

In the molecular biology era, the key biological drivers in nine published prognostic signatures were genes involved in proliferation, in addition to ER-signalling and HER2 activation pathways (Wirapati et al. 2008). Recent works from the Perou group, based on the existing 52 gene expression signatures, identified key drivers of proliferation in luminal breast cancers (*FGD5, METTL6, CPT1A, DTX3, MRPS23, EIF2S2, EIF6* and *SLC2A10*) which are uniquely amplified in patients with highly proliferative luminal breast tumours, and could be putative therapeutic targets (Gatza et al. 2014).

Proliferation assessment (IHC-based or not) in breast cancer is used to estimate prognosis by discriminating luminal A from luminal B subtype and consequently to guide the choice of chemotherapy in hormone receptor-positive, HER2-negative breast cancer. Proliferation reflects tumour aggressiveness and gives information for the identification of patients at risk of early relapses.

Proliferation evaluation does not bring any added value to the management of HER2-positive, triple negative, or metastatic disease (Aleskandarany et al. 2012; Van Poznak et al. 2015).

Therefore the degree of tumour cell proliferation is of paramount importance in ER-positive, HER2-negative breast cancers. This chapter is aimed to describe various tools for assessment of proliferation in breast cancer, with a special focus on Ki67 and the commercially available molecular signatures.

7.2 Mitotic Index/SBR Grade

Mitotic count or mitotic index is one of the three features evaluated in the Elston and Ellis modification of Scarff, Bloom and Richardson histologic grading (Rakha et al. 2008). The mitotic count score criteria vary depending on the field diameter of the microscope used by the pathologist (score 1: ≤ 3 mitoses/mm^2, score 2:4–7 mitoses/mm^2, score 3: ≥ 8 mitoses/mm)^2. The pathologist counts mitotic figures within 10 consecutive high-power fields (HPF, usually defined as the combination of 10× eyepiece and 40× objective). When using a HPF of 0.50 mm diameter, the criteria are as follows: (i) score 1: ≤ 7 mitoses per 10 HPF, (ii) score 2:8–14 mitoses per 10 HPF, (iii) score 3: ≥ 15 mitoses per 10 HPF. This is the oldest method to evaluate tumour proliferation. It has been proven to be prognostic of breast cancer-related death (reviewed in Beresford et al. 2006). Nevertheless, it is a subject of considerable variations depending on the thickness of the tissue section, fields chosen (mitotic counts are usually highest at the periphery of a tumour), type of microscope used, delay in fixation time, and observer's experience in the identification of mitotic figures (hyperchromatic, karyorrhexic, or apoptotic nuclei should not be considered as mitotic figures). Furthermore, the duration of the mitotic phase is variable, consequently mitotic count is not always strictly correlated to the proliferation rate in a linear fashion.

7.3 S-Phase Fraction and the Related Tools

The S-phase fraction ("the S-phase") corresponds to the measurement of the fraction of tumour cells engaged in DNA synthesis.

- **Tritiated thymidine** (3HTdR) **labelling index** (LI) was the first method used to evaluate the S-phase fraction. This method measured the incorporation of 3HTdR (a DNA precursor) into the dividing cells. The method required the use of fresh material and was time-consuming as autoradiography was performed on slides, usually several weeks after 3HTdR incorporation. The LI corresponds to the fraction of tumour cells (percentage) labelled by black nuclear dots. If 3HTdR incorporation was performed with cells in suspension, consequently, the isotopic emission is measured by a scintillation beta-counter. The 3HTdR LI tends to be much higher than the mitotic count because the cells stay longer in the S-phase than in the M-phase. The 3HTdR is extremely accurate, reproducible, however it not suitable for a routine use (requirement of fresh tissue, use of radioactive material, long assay duration).
- **5-bromodeoxyuridine (BrdU)** incorporation and its immunohistochemical detection was developed as specific assay for detection of DNA replication, avoiding the use of radiography and radioactive products (Gratzner 1982). BrdU assay showed comparable results to the 3HTdR assay. However, fresh and thin viable tissue is required and endogenous thymidylate activity has to be blocked.
- **Flow cytometry** is a technique that consists of measurement of various parameters while a suspension of cells flows through a beam of light past stationary detectors. The instrument focuses hydrodynamically a cell suspension in a sample chamber and passes single cells through a light source, usually a laser. The light scattered at various angles by the cells is registered by detectors and converted to electronic signals, which are then digitized, stored, and analysed by the computer to produce a histogram. This technique allows the analysis of 5000–10,000 cells per second. Flow cytometry can be used to analyse DNA content (DNA ploidy). Depending on their DNA content, neoplasms are divided into diploid and aneuploid. Diploid tumours have a major population with the normal diploid DNA value. Aneuploid tumours are those having a major cell population with a DNA content other than diploid. The **DNA index** (**DI**) is the ratio of the DNA content of the aneuploid peak to the DNA content of the diploid peak. The hyperdiploid fraction is the percentage of cells above the upper boundary of the diploid population and constitutes a measure of the S-phase or proliferative fraction of a cell population (S-phase fraction or SPF). Flow cytometry measurements of SPF have been shown to correlate with mitotic counts, histological grades and 3HTdR LI. The prognostic value of S-phase measurement has been shown in various retrospective studies (reviewed in Beresford et al. 2006). The flow cytometry method has two major limitations: (i) the fact that the stromal cells are also present in the population of cells being evaluated, thus the results do not solely reflect the malignant component; (ii) the requirement of fresh tissue, not suitable for a large spread of the technique.

7.4 Nuclear Antigens

Immunohistochemical (IHC) detection of nuclear antigens closely related to proliferation offers a unique opportunity to democratize the evaluation of tumour proliferation on formalin-fixed, paraffin-embedded (FFPE) tissue sections or on cytology specimens. Ki67 IHC assay is the most popular among those techniques and will be addressed at the end of this paragraph.

- **Phosphorylated histone H3** (PhH3) is expressed in the cells in mitotic phase. PhH3 is a nuclear core histone protein that is a component of chromatin. Its phosphorylation

at Serine 10 and Serine 28 is implicated in chromosome condensation and cell cycle progression during mitosis and meiosis (Lee et al. 2014). Thus number of cells expressing PhH3 should theoretically correlate with mitotic count. Therefore PhH3 has emerged as a potential IHC marker of mitotic activity and consequently of proliferation. Several reports showing positive correlation between mitotic and PhH3 counts have been published (Beresford et al. 2006). Due to lack of correlation between PhH3 and other markers of proliferation, PhH3 is currently considered more as an aid to the assessment of mitotic count than as a true proliferation marker (Dessauvagie et al. 2015).

- **Proliferating cell nuclear antigen** (PCNA) is an auxiliary protein of DNA polymerase delta. It seems to be essential for DNA synthesis and is expressed in high concentrations during the cell cycle. PCNA is also involved in DNA repair processes. PCNA correlates poorly with the Ki67 labelling index and mitotic count so is of more limited use in assessing proliferation and has become a dead letter (Leonardi et al. 1992).
- **Mitosin**, a nuclear phosphoprotein expressed in the late G1, S, G2, and M phases of the cell cycle but not in G0 has been evaluated as a substitute to S-phase (Clark et al. 1997). Good correlation was observed with the S-phase fraction, without a correlation with overall survival (Clark et al. 1997). This marker is no longer used in breast cancer. Recent publications have evaluated its accuracy as a prognosis marker in astrocytoma (Varughese et al. 2016).
- **Cyclins and cyclin-dependent kinases**: Progression through the cell cycle is dependent on the interactions between cyclins and cyclin-dependent kinases (CDKs). Cyclins are proteins which expression varies 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. They are, therefore, useful markers of the proportion of cells in a given phase of the cell cycle at any time. High expression of either cyclin A or cyclin E is associated with poor prognosis in breast cancer (Kuhling et al. 2003). Amplifications of the cyclin D1 gene (*CCND1*), found in 40 % of luminal breast cancers, and of the CDK4 gene (*CDK4*) are linked to hormone receptor-positivity and there is some evidence of a relationship between high levels of expression/amplification and poor prognosis (Roy et al. 2010). Correlations between expression of various cyclins and Ki67-based measurements of proliferation have been demonstrated in breast cancer (Beresford et al. 2006).
- **Inhibitors of the CDKs** can also be studied using immunohistochemical techniques: p16INK4a, p21 and p27 bind to and inhibit the activity of cyclin-CDK2 or -CDK4 complexes. Thus they control 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 (Catzavelos et al. 1997; Tsuchiya et al. 1999).

In the light of new drugs targeting CDK4/6 such as palbociclib (Roberts et al. 2012), several markers appeared to be potential candidates for response predictors, including retinoblastoma (Rb) protein loss or phosphorylation, inactivation of CDK4/6 inhibitors and amplification of genes for cyclins or CDKs. Inactivation of *RB1* appears to predict resistance to CDK4 and CDK6 inhibitors, but two of the most promising biomarkers, loss of *CDKN2A* (coding for p16INK4a) and gains of *CCND1* (coding for cyclin D1), failed to predict a benefit for palbociclib in ER-positive breast cancer in the PALOMA 1 trial (Finn et al. 2015). More studies are needed to evaluate putative biomarkers to better select patient eligible for CDK4/6 inhibitors (Carey and Perou 2015).

- **Argyrophilic nucleolar organiser regions** (AgNORs) are composed of non-histone proteins associated with loops of DNA transcribing to ribosomal RNA (Pich et al. 2000). The

number and size of AgNORs can be assessed by a silver-based staining of the tumour tissue. AgNORs are being aggregated and segregated during the cell cycle. Immediately after mitosis the NORs are dispersed through the nucleus and the nucleolus is not readily apparent. AgNOR staining reveals a large number of dots. The AgNOR count should be higher in cells in late G2 or early G1 when the NORs are segregated and they are more easily discernible. The major caveat with AgNOR assessment is that their number can also be elevated in benign proliferations so an elevated AgNOR count is not per se diagnostic of malignancy. The use of image analysis has improved the specificity of the AgNOR assay (Beresford et al. 2006). 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 in breast cancer (Winzer et al. 2013). Some authors have suggested to use AgNOR staining as an alternative measure of tumour proliferation (Raymond and Leong 1989; Canepa et al. 1990). Nevertheless, the AgNOR assay requires use of image analysis, so is introduced only in some laboratories, for research purposes.

- **Topoisomerase II (topoII)** is a nuclear enzyme which breaks and joins DNA strands. The isoform topoIIα is a marker of cell proliferation and also the molecular target for the anthracycline class of chemotherapy drugs commonly used in breast cancer treatment. Assessment of topoIIα expression by IHC has been shown to highly significantly correlate with tumour proliferation rate measured by SPF (Jarvinen et al. 1996) or by Ki67 labelling (Depowski et al. 2000; Misell et al. 2005), giving the information on the number of cycling tumour cells. High topoIIα expression is associated with an aggressive tumour phenotype, however the topoIIα IHC assay has not been developed for clinical use.
- **Thymidine kinase 1** (TK1) is an enzyme involved in phosphorylation of deoxy-thymidine during DNA synthesis. TK1 is expressed in the cytoplasm and activated at late G1 phase of the cell cycle. TK1 can be detected by IHC or ELISA; its expression is high in proliferating and malignant cells, but low or absent in quiescent cells. High levels of TK1 activity are associated with poor prognosis of breast cancer (Spyratos et al. 2002). This marker is currently used only for research approaches; the IHC assays for in vitro diagnostic use (IVD) are not developed.

7.5 Ki67

- **Background**: Ki67 index is the most developed and popular marker of proliferation, although with obvious flaws. Ki67 is a non-histone nuclear cortex protein, involved in the early steps of polymerase I-dependent ribosomal RNA synthesis. It was first identified by Gerdes et al in 1983 in a Hodgkin lymphoma cell line (Gerdes et al. 1983), then named Ki after Kiel University and 67 after the clone number of the antibody able to detect it. The gene coding for Ki67 (*MKI67*) is located on chromosome 10q25-ter and organized in 15 exons and 14 introns. Exon 13 contains sixteen Ki67 repeats including a highly conserved motif of 66 bp, named the Ki67 motif (Duchrow et al. 1996). The Ki67 protein is expressed in the cell nucleus during the G1, S, G2 and M phase of the cell cycle, but not in the G0 cell quiescent state. In the interphase the Ki67 protein is localized in the dense fibrillary components of the nucleolus. During mitosis it gets associated with the periphery of the condensed chromosomes. The Ki67 protein expression varies throughout the different phases of the cell cycle, being at the peak level during mitosis. While the function of the Ki67 protein is not completely elucidated, there is evidence that it has a role in cell division and ribosomal RNA synthesis. Ki67 index represents a percentage of tumour cells labelled with an anti-Ki67 antibody, in a IHC assay. It can serve as an

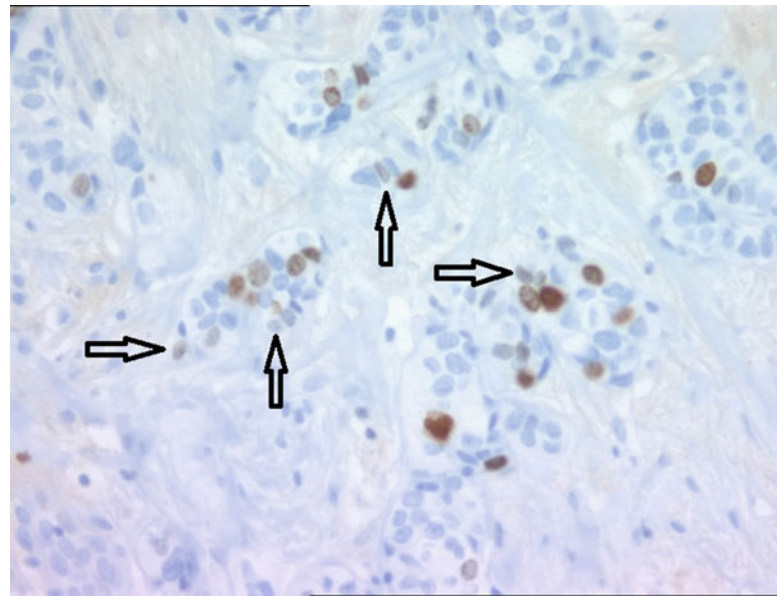

Fig. 7.1 Immunohistochemical detection of Ki67 using the MIB-1 clone (X400). Any intensity of nuclear staining indicates a Ki67-positive cell (*black arrows* show light brown positive nuclei)

alternative to mitotic index and correlates with increasing tumour grade. But Ki67 does not correlate well with PhH3 (r = 0.79) or mitotic score (r = 0.83) as reported by Lee et al. (2014) in a series of breast cancers. This confirms that PhH3 and Ki67 express distinct biological information and should be treated separately.

Despite massive literature addressing the caveats of Ki67 as an accurate biomarker for prognostication in early breast cancer (reviewed in de Azambuja et al. 2007; Yerushalmi et al. 2010; Dowsett et al. 2011; Luporsi et al. 2012), Ki67 is a popular and cheap biomarker in breast cancer, widely used to assess proliferation, and especially in segregating luminal A from luminal B tumours.

7.5.1 Analytical Validity

Lack of standardization impacts the analytical validity of Ki67. An international group of pathologists, clinicians and biologists was convened to examine data available upon Ki67 as a biomarker in early breast cancer and to propose guidelines (Dowsett et al. 2011). Several antibody clones, like MIB-1, MM-1, Ki-S5 and SP6, have been tested for Ki67 detection by IHC on FFPE tissue sections. The most popular and most widely used antibody is the MIB-1 clone.

As for any immunodetection, several **pre-analytical issues** such as time to fixation, type of fixative, duration of fixation and storage of slides with unstained tissue sections might adversely affect Ki67 expression assessment (reviewed in Dowsett et al. 2011). Eventually, the guidelines for tissue handling, which are already in place for ER immunohistochemical assessment (8–72 h of neutral buffered formalin fixation) (Hammond et al. 2010), can be considered for Ki67 IHC. Fortunately, Ki67 is one of the most robust biomarkers assessed by IHC, showing relatively consistent signals in tissue specimens across a range of conditions used in routine fixation, tissue processing, and IHC staining procedures.

Analytical steps are quite classical. Of note, protease and low pH methods for antigen retrieval should be avoided. Immunohistochemistry for Ki67 results in a nuclear staining. Any intensity of nuclear staining indicates a Ki67-positive cell (Fig. 7.1). Therefore it is important to have the counterstaining optimized, because, if it is weak, might result in an overestimation of the Ki-67 index.

How to count? The post-analytical phase of Ki67 IHC assay is the most critical one. The poor reproducibility reported for Ki67 scoring mainly resulted from a lack of consensus about which area of the tumour should be assessed, i.e. tumour invasive edge, a whole tumour section, or the hot spots (i.e. the areas of the highest proliferative activity). The international Ki67 in Breast Cancer Working Group provided guidelines covering also the Ki67 scoring (Dowsett et al. 2011) (Box 7.1). In brief, it is recommended to assess Ki67 either on core biopsies or on full-face tumour tissue sections. At least three HPFs should be selected to represent the spectrum of staining seen on the initial overview of the entire section. The invasive edge of the tumour should be counted and hot spots included in the overall score (Fig. 7.2). The Ki67 score or index should be expressed as the percentage of positively stained cells among the total number of invasive cancer cells in the area scored.

Overall, the International Ki67 in Breast Cancer Working Group concluded that measurements of proliferation could be important

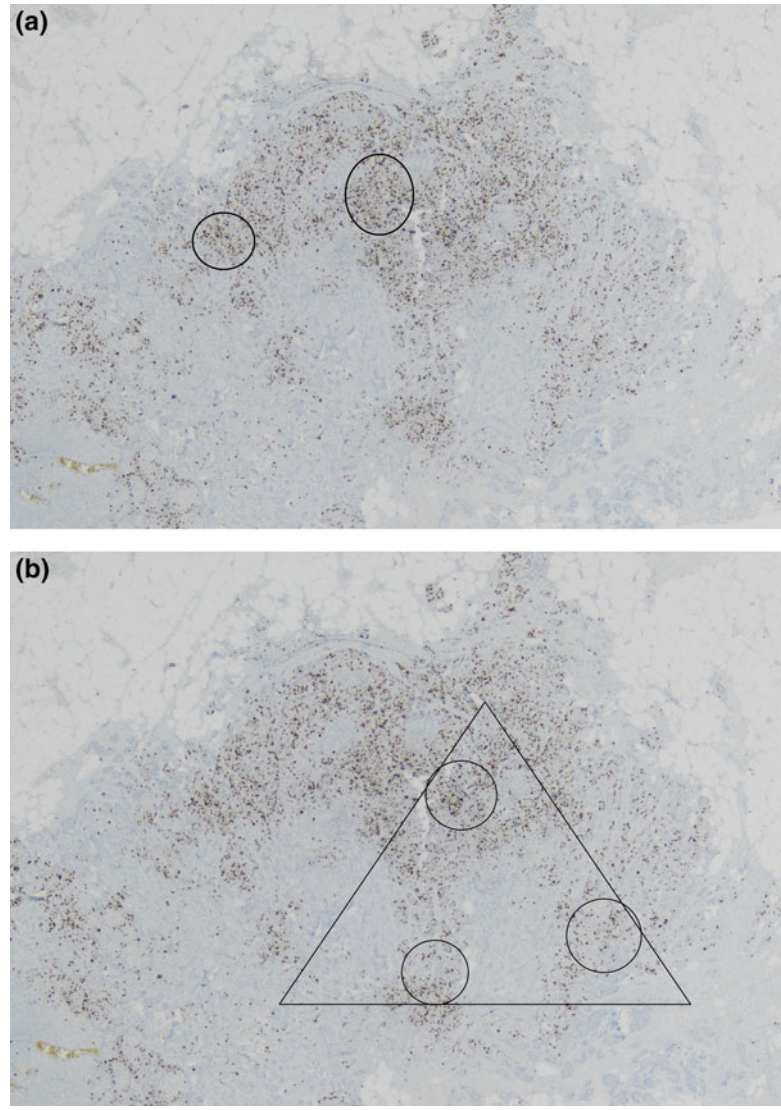

Fig. 7.2 Ki67 scoring. **a** Hot spots method: the evaluation is performed in the area with the highest number of positive nuclei (hot spot) (*black circle*). **b** Three high power fields including a hot spot: at least three HPFs should be selected to represent the spectrum of staining seen on the initial overview of the entire section. The invasive edge of the tumour should be counted and hot spots included in the overall score

both in standard clinical practice and, particularly, in clinical trials. Ki67 assessed by IHC using monoclonal antibody MIB-1 has the largest body of literature support. Standardization efforts have recently been made to improve the reproducibility of quantitative IHC assessment of Ki67 between different laboratories and observers, particularly with regards to the intermediate levels of Ki67 expression (Polley et al. 2013, 2015). The intra-class correlation coefficient (ICC) corresponding to the percentage of variance that is derived from the biomarker (i.e. Ki67) has to be as high (close to 1) as possible (otherwise the variance is due to the variation in interpretation). The International Ki67 in Breast Cancer Working Group showed that, with training and guidelines, the ICC for Ki67 went from 0.71 (95 % CI 0.47–0.78) to 0.92 (95 % CI 0.88–0.96). A quality assurance study from the Swiss working group of breast and gynaecological pathologists (Varga et al. 2012) evaluated the Ki67-based proliferative fraction in grade II breast carcinomas by different methods, for example, by eyeballing or by counting in self-selected versus the preselected areas. The reproducibility was good for low and high Ki67 indexes, but assessment of mid-range Ki67 was impaired by high inter- and intra-observer variability (Varga et al. 2012). The use of computer-assisted automated scoring proved to be helpful to standardize the assessment of Ki67 in breast cancer specimens in the GeparTrio trial and was well correlated with clinical endpoints (Klauschen et al. 2015). Finally, for the intermediate levels of Ki67 index, validated multigene assays could be a good re-test option (Goldhirsch et al. 2013; Coates et al. 2015).

7.5.2 Clinical Validity—Prognostic or Predictive?

Various meta-analyses (reviewed in de Azambuja et al. 2007; Yerushalmi et al. 2010; Dowsett et al. 2011; Luporsi et al. 2012; Andre et al. 2015) showed an independent prognostic value of Ki67 index for node-negative, ER-positive breast cancer [and to a lesser extent for the node-positive one (Andre et al. 2015)]. A study from the European Institute of Oncology showed that high Ki67 values (≥ 32 %) predict the benefit from cytotoxic chemotherapy addition in 1241 patients with luminal breast cancer and 1–3 axillary lymph nodes (Criscitiello et al. 2014). Nevertheless, other studies showed either a modest predictive value for chemotherapy benefit in node-positive patients [in PACS01 trial, for docetaxel addition (Penault-Llorca et al. 2009)], if any [such as in the BCIRG001 trial (Dumontet et al. 2010)]. In the neoadjuvant setting, high Ki67 index predicted for complete pathological response (pCR) in a large number of studies (de Azambuja et al. 2007; Luporsi et al. 2012; Denkert et al. 2013). Furthermore, Ki67 evaluation showed an important clinical utility as a pharmacodynamic or clinical endpoint for neoadjuvant treatment, namely for endocrine therapy (Jones et al. 2009). The largest study of post-neoadjuvant chemotherapy Ki67 index prognostic value in breast cancer, GeparTrio, distinguished three patient groups according to the Ki67 index level (0–15 vs. 15.1–35 vs. >35.1 %) (von Minckwitz et al. 2013). The low Ki67 group had an outcome comparable to the pCR group, while the high Ki67 group had a significantly higher recurrence and death risk compared to the low or intermediate Ki67 group. Taken together, the post-therapy Ki67 index level could provide additional prognostic information in the ER-positive breast cancer where pCR shows a limited prognostic value, whereas in the ER-negative cancer the post-neoadjuvant Ki67 does not have a stronger prognostic power than pCR (von Minckwitz et al. 2013). Decrease in Ki67 index is now being explored as the primary endpoint for pre-surgical trials with CDK4/6 inhibitors, like the Monaleesa-1 trial (NCT01919229).

- **Biomarkers combining Ki67 index with other parameters**: Ki67 index has been integrated into several mathematically derived parameters, which were tested as predictors of various features in breast cancer. In the following text we will highlight the most important ones:

Preoperative Endocrine Prognostic Index (PEPI) (Ellis et al. 2008) was generated using data of the P024 neoadjuvant endocrine breast cancer therapy trial in which prognostic relevance for recurrence-free survival (RFS) and overall survival (OS) was independently evaluated for five post-therapy tumour features: pathological size, pathological node status, ER status, histological grade and Ki67 index. The levels of Ki67 index were expressed in the form of natural logarithm-transformed intervals. PEPI was further constructed as a score representing an arithmetic sum of risk points assigned to each mentioned feature, according to its hazard risk estimate (Ellis et al. 2008). That way PEPI score distinguished three categories of significantly different risk for breast cancer-induced death. The PEPI score was independently validated on 203 patients included in the neoadjuvant IMPACT trial (Ellis et al. 2008) and is now being prospectively tested in the on-going ALTERNATE trial, conducted by The Alliance of Clinical Trials in Oncology, to identify patients with a very low recurrence risk after neoadjuvant endocrine therapy (Suman et al. 2015).

Residual Proliferative Cancer Burden (RPCB) (Sheri et al. 2015) was obtained by applying a formula that generates a sum of relative event rates for post-therapy Ki67 index and the Residual Cancer Burden (RCB) developed by Symmans et al. (2007). Its prognostic value for time to recurrence was evaluated in a cohort of 220 breast cancer patients treated by neoadjuvant chemotherapy. RPCB, classified into tertiles, was able to distribute the patients into groups with significantly different RFS and OS rates after a 5-year median follow-up (Sheri et al. 2015).

IHC4 is an IHC-based assay of four markers including Ki67, which has been shown to predict residual risk of distant recurrence in patients on adjuvant endocrine therapy in the ATAC trial as robustly as the recurrence score from OncotypeDX® (Cuzick et al. 2011). Recently, Engelberg and colleagues published a web-based pathologist training tool named "Score the Core" to improve the reproducibility of IHC4 scoring and thus eventually increase its clinical use (Engelberg et al. 2015).

MAGEE equation-based recurrence score (MS) is based on tumour pathological characteristics (SBR grade, H-scores for ER and PR, HER2, Ki67 index and tumour size) and can be used to estimate the Oncotype DX® recurrence score (RS), using the Magee equation (http://path.upmc.edu/onlineTools/ptvr.html) (Klein et al. 2013). The concordance between MS (tiered score) and RS was 98.6 %, when the intermediate category of MS was eliminated, but dropped to 54.3 % when the total populations were included in the comparison. Consequently, MS may be used instead of the actual Oncotype DX® RS, if the estimated MS is clearly high or low.

To summarize the complexities in evaluating the clinical utility of Ki67 in breast cancer, Denkert et al. (2015) have highlighted three different groups of tumours (quoted):

(a) Low proliferating tumours are not responding to chemotherapy but have a good prognosis anyway (low Ki67 linked to good outcome)
(b) In those high proliferating tumours that are therapy sensitive, high Ki67 is linked to an increased chance of pCR and improved survival (high Ki67 linked to good outcome)
(c) In contrast, in high proliferating tumours that are chemotherapy or hormone therapy resistant, increased Ki67 is linked to reduced survival (high Ki67 linked to poor outcome).

This suggests that, in the adjuvant setting, it is always very difficult to separate prognostic from predictive value of the Ki67 index. However, in the neoadjuvant setting, the pre-treatment Ki67 index is predictive and the post-treatment one is prognostic.

- **Clinical utility of the Ki67 index**: In breast cancer, Ki67 index is mainly used to discriminate luminal A from luminal B tumours in the ER-positive, HER2-negative breast cancers and consequently to guide the choice of chemotherapy, versus hormonotherapy alone. As mentioned by the St. Gallen expert Panel in 2015, "the distinction between strongly endocrine responsive, low proliferation, good prognosis 'luminal A-like' and less endocrine responsive, higher proliferation, poorer prognosis 'luminal B-like'

(HER2-negative) tumours could be derived from IHC tests for ER, PgR and Ki67, though the use of Ki67 required knowledge of local laboratory values" (Coates et al. 2015).

Does an ideal cut-off exist for Ki67? Despite all the lack of standardization and the variability in the cutpoints used to define a high Ki67 index (from 5 to 34 % or more), prognostic or predictive value of Ki67 index has been demonstrated in a majority of studies (Denkert et al. 2015). The St. Gallen consensus 2009 (Goldhirsch et al. 2009) proposed three categories: low (≤ 15 %), intermediate (16–30 %) and high (>30); St. Gallen 2011 (Goldhirsch et al. 2011) held for two categories with a cut-off of 14 % between luminal A and luminal B; St. Gallen 2013 (Goldhirsch et al. 2013) changed the cutpoint to 20 % with the option to use local laboratory values. In 2015, "a majority of the Panel was prepared to accept a threshold value of Ki67 within the range of 20–29 % to distinguish 'luminal B-like' disease" (Coates et al. 2015). Interestingly, a recent meta-analysis (Petrelli et al. 2015) of 41 studies, encompassing more than 64,000 patients, addressed specifically the major issue we have with the Ki67 clinical utility: which Ki67 cut-off provides the strongest prognostic information in early breast cancer (except in the neoadjuvant setting)? In 25 studies, available for analysis of the Ki67 cut-off significance for overall survival, the cut-off of 25 % was significant for prediction of OS (HR = 2.05, 95 % CI 1.66–2.53, $p < 0.00001$). Moreover, in the ER-positive population, this 25 % cut-off was also significant (HR = 1.51, 95 % CI 1.25–1.81, $p < 0.00001$). However, because of the complexity of the significance of Ki67 in different settings, reaching a unique cutpoint for Ki67 is likely idealistic.

- **Conclusion on Ki67**: Although not the most robust prognostic or predictive marker in breast cancer, Ki67 index is an additional piece of information that may be used in clinical decision making, provided the physician understands the limitations of the test and the test result. Ki67 IHC is widely available and less expensive than a multigene assay. Low Ki67 index (<15 %) is associated with good prognosis, whereas the high values (≥ 25 %) are likely predictive of chemosensitivity. The "grey zone" between 15 and 25 % might require either a second assessment by another pathologist, by image analysis, or use of multigene assays.

7.6 Molecular Signatures

In the early 2000s, several multigene signatures were developed [MammaPrint® (Agendia, the Netherlands), 76-gene signature (Veridex, USA), Oncotype DX® (Genomic Health, USA)] by searching, without a priori biologic assumption, for gene expression profiles associated with clinical outcome of breast cancer. Simultaneously, other signatures were developed in a "bottom-up" fashion by interrogating genes associated with a specific biologic process such as histologic grade [Genomic Grade Index or GGI® (Qiagen, the Netherlands)], wound healing, or invasiveness (reviewed in Wirapati et al. 2008). Although all demonstrate additional prognostic value, those gene expression signatures did not have many genes in common. They are described in details elsewhere in this book. The genes selected are implied in different biologic processes of breast cancer carcinogenesis e.g. cell cycle, invasion, metastasis, angiogenesis, immune response and, for some of them, in ER-, PR- and HER2-related pathways. The common denominator of all commercially available multigene assays are the proliferation genes, and it is believed that the group of proliferation-associated genes has the biggest impact on breast cancer prognosis (Mook et al. 2010). Proliferation-related (cell cycle) genes are highly represented in the available multigene assays: Oncotype Dx® has 5 proliferation-related genes, out of total 16: *MKI67*, *AURKA* (formerly *STK15*), *BIRC5* (coding for survivin), *CCNB1* and *MYBL2*; the 70-gene signature known as Mammaprint® contains 19 proliferation-related out of 70 total genes, the 76-gene signature (Rotterdam) has 16 out of 60 and the GG has 89 out of 98. Thus proliferation is the driving force

of prognostic information provided by those signatures. When only proliferation genes were used, the overall performance of the mentioned signatures was not reduced. In contrast, when proliferation genes were removed, the non-proliferation partial signatures showed reduced performance in giving the prognostic information. Consequently, this important weight of proliferation genes implies a strong time dependence of the prognostic information provided by the signatures, informative mainly for early (<5 years) recurrences.

Then a second generation of gene expression signatures was developed, with Endopredict® (Myriad Genetics, USA) and Prosigna® (Nanostring technologies, USA) signatures. Like OncotypeDX®, those commercially available tests are dedicated to ER-positive, HER2-negative breast cancers, node-negative or positive for up to three nodes. Endopredict® is a RT PCR-based test of 12 genes, with three proliferation-related genes (*UBE2C*, *BIRC5*, *DHCR7*), 5 ER-related genes, 3 normalization genes and one DNA control gene (Filipits et al. 2011). The results are given in a binary fashion (high risk vs low risk) with the Endopredict score (EP) or the Endopredict Clinical score (EP Clin) by adding tumour size and nodal status. Proliferation gene module predicts for early distant recurrence and oestrogen-related gene module for late (>5 years) recurrence). Prosigna® test provides the PAM50 profile (50 target genes plus eight normalization genes) of the intrinsic classification plus a 19 proliferation-associated gene expression module (*CCNE1, KIF2C, PTTG1, TYMS, KNTC2, CDCA1, MELK, CEP55, HSPC150, EX01 CCNB1, RRM2, UBE2C, CDC6, PHGDH, MYBL2, MKI67, CDC20, ORC6L, MYC*) along with tumour size (Nielsen et al. 2014). The test gives a risk of recurrence (ROR) score (with two different scales depending upon the nodal status), risk category (low, intermediate and high), and intrinsic subtype (luminal A/B, HER2-enriched, basal-like).

The capacity of gene signatures to predict late relapse in ER-positive breast cancer has been evaluated for Oncotype DX®, Prosigna®, and EndoPredict®, and all of them demonstrated independent correlation with late relapses, but the association was weaker than with early relapses. Thus, if proliferation-based gene expression signatures are strongly prognostic for early relapses, in ER-positive, HER2-negative breast cancers, they are suboptimal to predict late relapses, although that capacity is strong for PAM50 (Sestak et al. 2015) and encouraging data have been published for EndoPredict® and Oncotype DX® in post-menopausal women treated by hormonal treatment (Sestak et al. 2015; Tang et al. 2011; Alvarado et al. 2015).

7.7 Conclusion

Proliferation is a major biomarker in breast cancer, used for prognosis, prediction of treatment response, or both. Proliferation assessment is of paramount importance in ER-positive, HER2-negative breast cancers for guiding the choice of treatment. The most important methods for proliferation assessment in breast cancer are summarized in Table 7.1. In summary, mitotic index gives an insight into proliferation while S-phase and other biomarkers are not routinely used. Ki67 is a popular and cheap biomarker in breast cancer, widely used for measuring and monitoring tumour proliferation in breast specimens, despite poor agreement on its precise clinical utility, analytical approaches, scoring methods, cut-offs, use as a continuous variable for decision making, and data handling approaches. Ki67 appears to be a marker of the continuous variable type, reflecting tumour biology. Coordinated international efforts have provided rules to standardize Ki67 assessment and enhance its reproducibility. The clinical utility of very low and very high Ki67 indexes is good. Ki67 index cut-off of 25 % has shown significance for prediction of overall survival. For the "grey zone" Ki67 index, multigene assays might provide useful information to guide patient management in the ER-positive, HER2-negative breast cancers.

Table 7.1 Most frequently used proliferation markers in breast cancer

Marker	Specimen	Method	Reporting	Clinical utility
Mitotic index (MI)	FFPE tissue, undissociated	H&E staining	Count per 10 HPF	Prognosis (RFS, OS) (Beresford et al. 2006; Rakha et al. 2008)
Tritiated thymidine labeling index (3HTdR LI)	Fresh viable tissue, (undissociated or in a single cell suspension)	Autoradiography or measurement of radioactivity (counter)	Fraction (% of labelled tumour cells) or counts per minute	Prognosis (RFS) (Paradiso et al. 1990; Nio et al. 1999)
5-bromodeoxyuridine labeling index (BrdU LI)	Fresh viable tissue, (in a single cell suspension or undissociated)	Flow cytometry or IHC	Fraction (% of labelled tumour cells)	Prognosis (RFS) (Meyer and Province 1994)
DNA content (DNA index, DI)	FFPE or fresh, in a single cell suspension	Flow cytometry	Ratio between DNA content of normal and the examined population	Prognosis (RFS); reviewed in Danielsen et al. (2015)
Phosphorylated histone 3	Any, most frequently FFPE undissociated	IHC	Count per 10 HPF	Putative prognostic marker (complement to MI)
Proliferating cell nuclear antigen (PCNA)	Any	IHC	Fraction (% of labelled cells)	Prognosis (RFS, OS) (Tahan et al. 1993; Haerslev and Jacobsen 1994; Stuart-Harris et al. 2008)
Cyclins (D1, E, A)	Any, most frequently FFPE undissociated	IHC or ISH	Histoscore (IHC), number of copies (ISH)	Prognosis (RFS, OS) (Lundgren et al. 2012; Xu et al. 2013; Roy et al. 2010; Gao et al. 2013; Klintman et al. 2013)
Topoisomerase 2α	FFPE tissue, undissociated	ISH	Number of copies	Prediction of response to anthracyclines (Press et al. 2011; Du et al. 2011)
Ki67	Any	IHC	Fraction (% of labelled tumour cells)	Prediction of response to taxane-based chemotherapy (de Azambuja et al. 2007; Luporsi et al. 2012; Criscitiello et al. 2014; Denkert et al. 2013), pharmacodynamic marker for neoadjuvant endocrine therapy (Jones et al. 2009), prognosis (RFS and OS) (Denkert et al. 2013; von Minckwitz et al. 2013)
Ki67-based mathematically-derived markers (PEPI, RPCB, IHC4)	FFPE tissue, undissociated	IHC and H&E staining	Scores	Prognosis (RFS) (Ellis et al. 2008; Sheri et al. 2015; Cuzick et al. 2011)

(continued)

Table 7.1 (continued)

Marker	Specimen	Method	Reporting	Clinical utility
Multigene assays (Mammaprint®, OncotypeDX®, EndoPredict®, PAM50/Prosigna®)	Fresh or FFPE, undissociated	RT-pCR, "digital" pCR (Nanostring technology)	Scores	Prognosis (RFS) (Filipits et al. 2011; Dubsky et al. 2013; Saghatchian et al. 2013; Sgroi et al. 2013; Gnant et al. 2014, 2015; Filipits et al. 2014; Sestak et al. 2015), benefit from adjuvant chemotherapy (Oncotype DX®) (Tang et al. 2011)

FFPE formalin-fixed, paraffin-embedded; *H&E* haematoxylin-eosin; *HPF* high power field, *RFS* recurrence-free survival; *OS* overall survival; *IHC* immunohistochemistry, *ISH* in situ hybridization

BOX 7.1: Recommendations for Ki67 assessment in breast cancer from the International Ki67 in Breast Cancer Working Group (Dowsett et al. 2011)

Preanalytical

- Core-cut biopsies and whole sections from excision biopsies are acceptable specimens; when comparative scores are to be made it is preferable to use the same type for both samples (e.g. in presurgical studies).
- Tissue micro-arrays are acceptable for clinical trial evaluation or epidemiological studies of Ki67.
- Fixation in neutral buffered formalin should follow the same guidelines as published for steroid receptors.
- Once prepared, tissue sections should not be stored at room temperature for longer than 14 days. Results after longer storage must be viewed with caution.

Analytical

- Known positive and negative controls should be included in all batches; positive nuclei of non-malignant cells and positive nuclei with mitotic figures provide evidence of the quality of an individual section.
- Antigen retrieval procedures are required. The best evidence supports the use of heat-induced retrieval most frequently by microwave processing.
- The MIB-1 antibody is currently endorsed for Ki67.

Interpretation and Scoring

- In full sections, at least 3 high-power (×40 objective) fields should be selected to represent the spectrum of staining seen on initial overview of the whole section.
- For the purpose of prognostic evaluation the invasive edge of the tumour should be scored.
- If pharmacodynamic comparisons must be made between core-cuts and sections from the excision, assessment of the latter should be across the whole tumour.
- If there are clear hot-spots, data from these should be included in the overall score.
- Only nuclear staining is considered positive. Staining intensity is not relevant.
- Scoring should involve the counting of at least 500 malignant invasive cells (and preferably at least 1000 cells) unless a protocol clearly states reasons for fewer being acceptable.
- Image analysis methods for Ki67 remain to be proven for use in clinical practice.

Data Handling

- The Ki67 score or index should be expressed as the percentage of positively staining cells among the total number of invasive cells in the area scored.
- Statistical analysis should take account of the log-normal distribution generally followed by Ki67 measurement.
- The most appropriate end-point in comparative studies of treatment efficacy or response is the percentage suppression of Ki67-positive cells.
- The most appropriate end-point for assessing residual risk of recurrence is the on-treatment proportion of Ki67-positive cells.
- Cut-points for prognosis, prediction and monitoring should only be applied if the results from local practice have been validated against those in studies that have defined the cut-off for the intended use of the Ki67 result.

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