CURRENT TOPICS IN BREAST PATHOLOGY

Ki67 assessment in breast cancer: an update

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Summary

Although immunohistochemical detection of the Ki67 antigen has been used for many years to assess cancer proliferation, this marker is still not recommended for routine use in clinical management of breast cancer. The major reason for this situation is a lack of a standardised procedure for Ki67 assessment as well as persistence of several issues of debate with regards to the Ki67 assay interpretation and the marker's clinical utility. Nowadays Ki67 assessment is principally used for estimation of prognosis and guiding the decision on adjuvant treatment choice, as well as for prediction of response to neoadjuvant treatment in ER+/HER2− breast cancer. In ER−/HER2+ and ER−/HER2− tumours, high post-neoadjuvant Ki67 index is associated with unfavourable prognosis. We review here the elements impacting analytical validity of the Ki67 immunohistochemical assay, the evidence of its clinical utility and the current recommendations for its use in breast cancer management.

INTRODUCTION

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.[1] The molecule was named Ki after the 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 organised in 15 exons and 14 introns. Exon 13 contains sixteen Ki67 repeats including a highly conserved motif of 66 bp, named the Ki67 motif.[2] Ki67 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 Ki67 is localised in the dense fibrillary components of the nucleolus. During mitosis it gets associated with the periphery of the condensed chromosomes. Expression of Ki67 varies throughout the cell cycle, reaching the peak level during mitosis. While the function of Ki67 is not completely elucidated, there is evidence of its role in cell division and ribosomal RNA synthesis.

For assessment of tissue proliferation, Ki67 expression is typically detected by immunohistochemistry (IHC) and reported as Ki67 index (often reported only as 'Ki67'), which represents the percentage of labelled cells within the investigated cell population (in tumours, Ki67 index is a percentage of labelled tumour cells). Ki67 index does not correlate very well with phosphorylated histone H3 (PhH3) index or with mitotic index ($r = 0.79$ and $r = 0.83$, respectively) as reported by Lee *et al.* in a series of breast cancers.[3] This confirms that PhH3 and Ki67 give distinct biological information and should be treated separately. PhH3 is a nuclear core histone protein involved in chromosome condensation and cell cycle progression during mitosis and meiosis, and should be considered more as a potential marker of mitotic activity/count whereas Ki67 can serve as an alternative to the proliferative activity.

ANALYTICAL VALIDITY

Lack of standardisation 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.[4] Several antibody clones, like MIB-1, MM-1, Ki-S5 and SP6, have been tested for Ki67 detection by IHC on formalin fixed, paraffin embedded (FFPE) tissue sections. The most popular and most widely used antibody is the MIB-1 clone.

Pre-analytical phase

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 detection of Ki67 (reviewed in Dowsett *et al.*)[4]. The guidelines for tissue handling, which are already in place for oestrogen receptor (ER) IHC assessment (8–72 hours of fixation in neutral buffered formalin),[5] 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 procedures.

Analytical phase

The analytical phase is quite classical. Of note, protease and low pH methods for antigen retrieval should be avoided. IHC

for Ki67 results in a nuclear staining. Any intensity of nuclear staining indicates a Ki67 positive cell (Fig. 1). Therefore, it is important to have the counterstaining optimised; if weak, this might result in an overestimation of the Ki67 index.

Post-analytical phase

The post-analytical phase is the most critical. The poor reproducibility reported for Ki67 scoring has 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 has provided guidelines covering Ki67 scoring[4] (Box 1). In brief, it is recommended to assess Ki67 either on core biopsies or on full-face tumour tissue sections. At least three high power fields (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. 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 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. Standardisation 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.[6,7] The intra-class correlation coefficient (ICC), corresponding to the percentage of variance that is derived from a 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 increased from 0.71 [95% confidence interval (CI) 0.47–0.78] to 0.92 (95% CI 0.88–0.96).[6] A quality assurance study from the Swiss Working Group of Breast and Gynaecopathologists[8] evaluated the Ki67-based proliferative
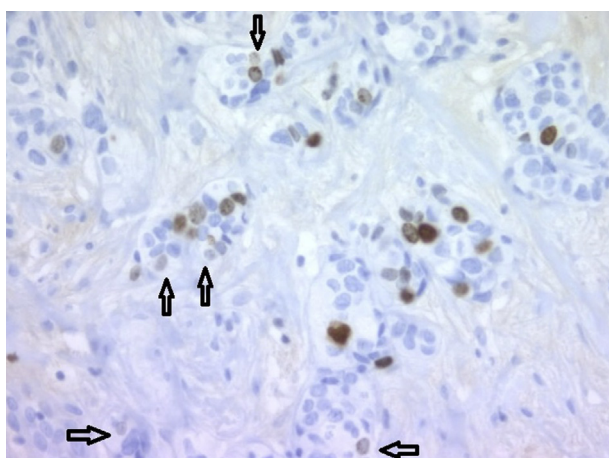

Fig. 1 Immunohistochemical staining for Ki67, using the MIB-1 clone (Dako), x40. Any intensity of nuclear staining indicates a Ki67 positive cell. Black arrows show light brown positive nuclei.

Box 1. Recommendations for Ki67 assessment in breast cancer from the International Ki67 in Breast Cancer Working Group[4]

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 reduction 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.