The expression, assessment, and significance of Ki67 expression in breast cancer: an update

Ayat Lashen[1,2,3] Michael S Toss[1,3,4], Suzan Ghannam[1,3,5], Shorouk Makhlouf[1,3,6], Andrew R Green[1,3], Nigel P Mongan[7,8] and Emad Rakha[1,2,3,9]

[1]Academic Unit for Translational Medical Sciences, School of Medicine, University of Nottingham, Nottingham, UK, [2]Department of Pathology, Faculty of Medicine, Menoufia University, Shebin El Kom, Egypt, [3]Nottingham Breast Cancer Research Centre, University of Nottingham, Nottingham, UK, [4]Department of Histopathology, Sheffield Teaching Hospitals NHS Foundation Trust Sheffield, United Kingdom, [5] Department of histology and cell biology, Suez Canal University, Ismailia, Egypt.[6] Department of Pathology, Faculty of Medicine, Assiut University, Assiut, Egypt. [7]School of Veterinary Medicine and Sciences, University of Nottingham, Nottingham; [8]Department of Pharmacology, Weill Cornell Medicine, New York, USA. [9]Pathology Department, Hamad Medical Corporation, Doha, Qatar.

Corresponding Author: Professor Emad Rakha

Department of Histopathology, Division of Cancer and Stem Cells, School of Medicine, The University of Nottingham and Nottingham University Hospitals NHS Trust, Nottingham City Hospital, Nottingham, NG5 1PB, UK.

Email: emad.rakha@nottingham.ac.uk

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ABSTRACT

Ki67 expression is one of the most important and cost-effective surrogate markers of assessing tumour cell proliferation in breast cancer (BC). Ki67 labelling index has prognostic and predictive value in patients with early-stage BC, particularly in the hormone receptor positive HER2 negative (luminal) tumours. However, many challenges exist for using Ki67 in routine clinical practice and it is still not universally used in the clinical setting. Addressing these challenges can potentially improve the clinical utility of Ki67 in BC. In this article, we review the function, immunohistochemical (IHC) expression, methods of scoring and interpretation of the results and addressed several challenges of Ki67 assessment in BC. The prodigious attention associated with the use of Ki67 IHC as a prognostic marker in BC resulted in high expectation and overestimation of its performance. However, the realisation of some pitfalls and disadvantages, which are expected with any similar markers, resulted in increasing criticism of its clinical use. It is time to consider a pragmatic approach and weigh the benefits against the weakness and identify factors to achieve the best clinical utility. Here we highlight strengthens of its performance and provide some insights to overcome the existing challenges.

INTRODUCTION

Uncontrolled cell proliferation is a hallmark of cancer, and tumour proliferative activity is one of the most thoroughly investigated cellular functions in breast cancer (BC) [1]. Therefore, different techniques have been used to estimate cellular proliferation, including bromodeoxyuridine (BrdU) and tritiated thymidine, which were considered the gold standard measures of cellular proliferation [2]. The percentage of cells in S-phase of the replicative cycle can also be measured by flow cytometry [3]. However, these techniques are not easily replicated in the clinical practice due to the complexity of requiring fresh tissue. Evaluation of proliferative activity using immunohistochemistry (IHC) offers an easy, cheap, and reliable method of assessing tumour cell proliferation in BC [4]. Although Ki-S1, topoisomerase IIα, phosphor-histone H3 (PHH3), proliferating cell nuclear antigen (PCNA), and minichromosome maintenance (MCM) proteins have been used to assess proliferation in BC (Table 1), Ki67 remains the most used in the clinical setting [5 6].

Ki67 is located in the cell nucleus where it only attached to the perichromosomal layer of dividing cell [5]. The IHC expression of Ki67 in BC strongly correlates with cellular proliferation which is the main determinant of tumour growth rate and is related to behaviour and response to cytotoxic chemotherapy [7]. The relationship between Ki67 expression and outcome of BC patients has been widely studied [6 8 9]. A meta-analysis of 43 studies involving 15,790 BC patients, confirmed that high Ki67 levels is associated with shorter survival in both lymph node positive or negative disease [10]. The use of Ki67 expression in BC was extended to differentiate luminal A from luminal B tumours [11]. Although its prognostic significance was mainly observed in the hormone receptor positive HER2 negative (luminal) BC, its role was also investigated in the triple negative and HER2 positive tumours [6 12]. The initial use of Ki67 IHC as a prognostic marker in BC have attracted a great deal of attention of the clinical community and recommendation for its routine use in the clinical setting. However, this high expectation of the performance of Ki67 have resulted in overestimation of its analytical validity and its clinical utility. This was followed by the realisation of some pitfalls and challenges, which are expected with any similar IHC markers. With time, opponents to its clinical value increased and overemphasised the expected weakness of performance. It is now the time to consider a pragmatic approach and weigh the benefits against the weakness and identify factors to achieve the best utility of Ki67 in

BC. In this review, we review the biology, expression, and clinical utility of Ki67 in BC and highlight strengths of its performance and provide some insights to overcome the existing challenges.

STRUCTURE AND FUNCTIONS OF Ki67

Ki67 is a labile non-histone protein, involved in the early steps of polymerase I-dependent ribosomal RNA synthesis [13] [14]. Ki67 is a nuclear antigen expressed in all proliferating cells, during cell cycle phases S, G1, G2, and M, but not in G0 [14]. During interphase, Ki67 is located in the nucleolar cortex and the dense fibrillar components of the nucleolus, whereas it becomes associated with the periphery of condensed chromosomes during mitosis. Ki67 levels are low in G1 and S phases with peak levels during mitosis especially metaphase, then sharply decreases during anaphase [15]. Similar to other cell cycle markers, Ki67 is affected by external factors such as cellular nutrient deprivation, which lead to underestimation of the number of cycling cells [16]. The half-life of the Ki67 antigen is between 1–1·5 hours, regardless of the stage of the cell cycle [17].

Ki67 is required during interphase for normal cellular distribution of heterochromatin antigens and for the nucleolar association of heterochromatin [18]. Ki67 binds with satellite DNA and its protein bound complexes to promote chromatin compaction [19].Ki67 is a key organiser of the chromosome periphery [20] and it is also essential for organisation of the perichromosomal layer, and its absence would affect the ability of daughter cells to synthesise protein building blocks and increase the rate of spontaneous cell death [20]. These features make Ki67 one of the best markers to assess cell proliferation for its crucial role in cell cycle. Figure 1 shows examples of Ki67-positive mitotic cells using IHC and Ki67 surrounding chromosomes (perichromosomal layer).

Ki67 ASSESSMENT

Ki67 antibodies for immunohistochemistry

Several antibodies are used for Ki67 antigen detection, including Ki67 and MIB1 monoclonal antibodies directed against different epitopes [21]. MIB1 has a higher sensitivity for detecting the Ki67 antigen [22]. Other antibodies against Ki67 are also available, such as SP6 which recognises the same epitope as MIB1, has been used to assess proliferation in BC [23 24]. However, some studies have shown that SP6 was not superior to MIB1 antibody with head-to-head comparison of both markers [24]. Therefore, MIB1 is the widely used antibody for Ki67 assessment.

Localisation and patterns of Ki67 IHC staining.

Ki67 is a nuclear protein (Figures 2&3) [25] and other non-nuclear staining patterns are not considered in the scoring [26] and their significance is still unclear [27 28]. Cross-reactivity with other proteins, technical artefact, or re-localisation of Ki67 within the cell are possible explanations [27]. There are different patterns of nuclear Ki67 expression observed in BC including homogeneous staining pattern of the nucleoplasm, a granular pattern, which stained nucleoli or granules of different size dispersed throughout the nucleoplasm and a mixed pattern that showed strongly stained granules against a diffusely positive background of a lower staining intensity [29]. The clinical relevance and impact of these distinct Ki67 staining patterns in BC remain poorly understood. In one study, it was reported that 80% of luminal BC have homogeneous nuclear staining, while granular staining pattern was observed in 18% of cases [30], and this pattern was associated with shorter survival compared with other Ki67 patterns. This can highlight that granular pattern could provide information about the patient's prognosis beyond what is given by the percentage of Ki67 expression alone. However, assessment of the pattern of staining in routine practice is challenging, difficult to be integrated with the percentage of expression and may result in confusion about the actual prognostic significance if used with the percentage of scoring. Therefore, in the routine practice assessment of the percentage of Ki67 expression regardless the pattern of expression can be considered adequate for scoring.

Intensity of Ki67 staining

The intensity of nuclear staining of Ki67 ranges from weak, moderate, to strong staining [31] and this may reflect the fluctuation of Ki67 levels in the different phases of cell cycle. In previous study, assessment of the intensity combined with the percentage of Ki67 expression calculated as H-score [30] showed strong associations with tumour

clinicopathological parameters including tumour size, and grade but not with patient outcome [30]. Therefore, the intensity of staining does not seem to add prognostic significance and nuclear positivity of any intensity should be considered in the scoring.

Heterogeneity of Ki67 staining

The inter-tumour heterogeneity of Ki67 staining across biological BC subgroups, especially in luminal tumours was previously studied [32]. Some BC types are characterised by low Ki67 expression such as tubular and invasive cribriform carcinoma, classical lobular carcinoma, and other grade 1 special type carcinomas while other tumour types show variable degree of expression from low to high such as no special type carcinoma (NST). These tumours also show intra-tumoural heterogeneity [33 34]. This has been explored using a Ki67 heterogeneity index (the difference between Ki67 expression levels in hotspot and global Ki67 expression in the whole tumour). A higher heterogeneity index is associated with parameters characteristic of aggressive tumour behaviour and poor outcome [30]. This may reflect the presence of highly proliferative clones in heterogeneous tumours compared to homogeneous tumours, in which the majority are low proliferative. This heterogeneity also supports the use of hotspots to assess Ki67 score which should represent the most proliferative part of the tumour rather than the average proliferative activity which will be lower than that of the hotspots.

Ki67 expression in needle core biopsies and surgical excision specimens

The concordance of Ki67 expression in core biopsies and full-face sections has been a point of interest. A recent systematic review involved 22 studies revealed a wide concordance kappa coefficient range from 0.2 to 0.7 between biopsy types. However, different methodologies and cut-offs used explains the results. Ki67 has been reported to be higher expressed in core than excision biopsies [35] which may be related to fixation issues, particularly in cohorts enriched with cases that had suboptimal fixation of surgically resected specimens. This result supported the recommendations of the International Ki67 in Breast Cancer Working Group (IKWG) for testing Ki67 on core biopsies in general. However, other authors [36] demonstrated that Ki67 is higher in the surgical specimen than in the paired baseline core biopsies, which is expected considered the intra-tumoural heterogeneity of Ki67 and the distribution of its expression in hotspots. We believe that the heterogeneity in Ki67 expression denotes

that full-face sections, under good fixative protocols, are the optimal platform to assess Ki67 properly and avoid the underestimation of Ki67 expression in heterogeneous tumours which are not uncommon. However, core biopsy can be similar or better than excision specimens in the assessment of Ki67 expression when the fixation of the excision specimen is suboptimal or in tumours with extreme end of the spectrum of expression (low or diffusely high expression) [37]. It is also the ideal method to test the tumour proliferative activity in the setting of neoadjuvant therapy as the residual tumours' proliferative activity can be influenced by the treatment effect.

Methodological challenges in Ki67 assessment

Pre-analytical setting: Similar to other IHC predictive markers in BC, several pre-analytical issues have been shown to affect Ki67 expression such as type and duration of fixation [38 39]. Optimal Ki67 results are obtained when 10% neutral buffered formalin is used. Prolonged fixation may cause reduction in Ki67 expression levels [40]. Importantly, long-term storage in paraffin affects the accuracy of Ki67 assessment as it is also more sensitive to antigen decay [41]. It was recommended that samples for Ki67 staining should be processed as the ASCO and CAP guidelines for hormonal receptors and HER2 to preclude many pre-analytical challenges [42].

Analytical and post-analytic issues of Ki67 assessment: The degree of inter-laboratory variability in Ki67 staining results from differences in staining methodologies, including staining platform, antigen retrieval, primary antibody, detection system, and counterstain [42]. However, this degree of inter-laboratory discordance is not different to estrogen receptor (ER) and progesterone receptor (PR) staining and other similar diagnostic and prognostic IHC markers in routine practice and should not be used as a major criticism against its clinical use.

There are many post-analytic issues of Ki67 assessment, whereas cell counting for Ki67 is the most challenging and may be impractical. Another caveat is assessment using hotspots or using an average value across specimens, counting non-tumour cells such as tumour infiltrating lymphocytes or non-invasive tumour, such as ductal carcinoma *in situ* (DCIS) which may contribute to scoring problems.

Scoring methods

IHC scoring is commonly based on the percentage of stained tumour cells reported as the Ki67-labelling index (Ki67-LI); however, less labour intensive Ki67 scoring methods have also been proposed [43]. The common scoring methods include assessing Ki67 in 500 or 1000 tumour cells, either in whole tissue sections or focussing in a highly stained 'hotspot' areas [6 44-48]. Others have used a global subjective assessment of Ki67 positivity by rapidly scanning and estimating the percentage of Ki67-positive nuclei at high power magnification (termed the "quick-scan" method) [49 50]. Some authors have also suggested scoring Ki67 in 200-400 cells in 10 high powered fields at 40x magnification [51] while others have recommended assessment of the whole tissue section at low power and record the overall average percentage score [52]. Recently, IKWG has suggested calibrated standardised visual scoring using online scoring applications [53]. However, it requires routine digitalisation of the stained sections which couldn't be available in all pathology laboratories. In a recent study, we determined that counting Ki67 positive cells among 1000 invasive breast tumour cells within the 'hotspot', showed the highest degree of consistency between multiple observers, and the highest hazard ratio predicting patient outcome when compared to other scoring methods [30]. However, this approach may be a time-consuming method in routine practice. Therefore, we tested a more practical method based on the visual estimation of Ki67 without counting within an average of 1000 cells considering the tumour cell density and the number of cells per specific areas in tumours with variable cellularity. In this approach, pathologists select a high-power field (HPF), dependent on the field diameter of the used microscope, and estimated the number of tumour cells per HPF in tumours with different cell density and assess the approximate number of HPFs that are required to count 1000 cells per case without the actual cell count i.e. (calibrate their fields). Then they can visually estimate the average percentage of Ki67 positive cells in the area that contain approximately 1000 cells or more without counting the cells (the average number of HPFs that contain >=1000 tumour cells is variable and range from (100 to1000 cells) based on microscope field diameter and the degree of tumour cellularity [30]. This method is likely to reduce scoring time and achieved high inter-observers concordance [30]. Similar standardisation of methodology has been adopted by the WHO group in counting mitotic figures per mm2 rather than solely on the 10 HPF which may vary in size significantly [54]. Calibration of the HPF of the specific microscope is also helpful in the accurate counting of mitotic figures that is already part of the Nottingham grading system.

Depending on the degree of the homogeneity of staining, previous studies advocated that Ki67 should be assessed in at least three randomly selected HPFs at 40x objective [6 55 56] or in two peripheral along with one central heterogeneous HPFs [57]. However, in heterogeneous staining, scoring is suggested to be in three HPFs from the tumour edges or hotspots [6]. However, adopting these approaches do not consider density of the different tumours, which has important impact on the assessment in Ki67 [30].

Ki67 cut-offs for clinical use

Consistent cut-off point is crucial when considering the prognosis for luminal BC patients. Cut-offs to distinguish “Ki67 high” from “Ki67 low” in luminal BC varied from 1% to 29%, which is one of the perceived limitations of Ki67 use in the clinical setting. In 2011, Saint Gallen Consensus Meeting defined the “low proliferation” tumours to have Ki67LI <14 % [58], which was proposed based on a study by Cheang and colleagues [59]. In 2013 Saint Gallen Conference, the majority voted that a threshold of ≥ 20% was indicative of “high” Ki67 status. However, in the St. Gallen conference of 2015, the majority favoured a cut-off value of 20–29% [60]. A previous meta-analysis of 64,196 patients showed that of cut-off of at least 25% has better discriminatory power compared to other cut-offs in luminal BC [61]. Another study included 8088 patients and used automated Ki67 scoring concluded that Ki67 >12 % had a worse 10-year breast cancer specific survival (BCSS) in ER positive BC patients [62]. Moreover, Ki67 has been compared with EndoPredict risk scores and Ki67 above 25% was found to show a strong overlap with high-risk EndoPredict test [63]. It seems that cut-off levels ranging from 10% to 30% have been the most common to dichotomise populations [64 65]. Al Eskandarany and his colleagues demonstrated that 10% cut-off for Ki67 could stratify BC with grade I and II into statistically significant prognostic groups [66]. Others recommended to use the median value of local laboratory as the cut-off [60]. The IKWG recently concluded that Ki67 index of 5% or less, or 30% or more, can be used to estimate prognosis in early BC [53]. However, there is still a large gap between 5% and 30% and a large percentage of patients would be involved in this zone. In our cohort of luminal BC patients (n=2641), less than 20% of patients had Ki67 expression <5% and less than 15% showed more than 30% Ki67 expression. This will classify a third of BC patients as a whole, while two-thirds of cases will remain in indeterminate zone, which limit the value of Ki67.

In TNBC, a 40% cut-off was considered an optimal cut-off to classify patient with greater risk of recurrence and death compared with patients with lower expression rates [67] others found that 30% is the relevant cut-off value for Ki-67 for prognosis of TNBC [68]. Although, different cut-offs for Ki67 were proposed in TNBC, its cohort-based and with limited prognostic value. While the extreme end of spectrum is helpful; choosing specific cut-off does not have impact on TNBC patients.

Also, Ki67 cut-off should be changeable according to the context of its use either diagnostic or prognostic. For example, 2% cut-off was used in atypical hyperplasia to predict the risk of developing BC [69]. Also, it was shown that patients with Ki67 >10% after pre-operative endocrine therapy were considered a candidate for further adjuvant treatments in the POETIC clinical trial [70]. Furthermore, 20% Ki67 cut-off was accepted by FDA for addition of CDK4/6 Inhibitor with endocrine therapy to luminal high risk BC patients [71]. In addition, it could be considered to predict the response of CDK4/6 Inhibitor [72]

Ki67 and mitosis

Ki67 and mitotic index (MI) are indicators of tumour proliferation rate and both are significantly correlated [6]. Analogous to Ki67, the advancing edge of the tumour, probably in the areas of highest mitotic counts, is likely to be representative of tumour clones that drive the biologic potential of the tumour [44 73]. Mitotic count, which can easily be assessed in a defined area on H&E-stained slides, represent the dividing cells while Ki67 IHC represent the cells in the cell cycle. Not all cells in the cell cycle will proceed to the mitosis phase and become committed to cell division. Therefore, MI may provide a more reliable representation of the tumour growth rate than KI67 IHC. In recent study [52], we assessed the proportion of BC cells in the cell cycle alongside mitosis and found that the mean proportion of BC cells in mitosis was 5%. This support scoring mitosis by counting the number of mitotic figures while scoring Ki67 by assessing the percentage of positive cells.

A standardised method of mitotic counts as part of the Nottingham grading system has been developed and is widely used. Although grade is one of the important prognostic parameters in BC that attracted a lot of attention, mitosis count as part of the 3 components of grade did not attract similar attention like Ki67 as a measure of

proliferation. This may be related to several factors including the low concordance rate among pathologists in assessing mitotic scores and the lack of evidence on its prognostic significance as a standalone parameter in BC. The prognostic significance of mitotic count in the clinical setting remains as a component of grade. However, after improved standardisation of mitotic count in BC and defining the area of counting rather than the number of HPF may improve concordance of assessment and it will be possible to develop mitotic cut-off of prognostic significance similar to Ki67 rather than the 3 scores of grade [74]. In contrast to MI that is subject to fixation factors, Ki67 can better represent the proliferative activity in tumours with suboptimal fixation and can provide an alternative for mitotic count during assessment of BC grade in sub optimally fixed tissue [66]. Core needle biopsy may be the optimal type of specimens to assess mitosis or Ki67 to avoid the impact of fixation issues and a previous study showed that higher mitotic scores associated with worse outcomes using core needle biopsy [75]. However, we showed previously that the concordance between mitotic scores in core biopsy and surgical sections was (63%; κ-value = 0.25) and there was underestimation of mitoses in the needle biopsy specimen which leads to grade discrepancy in some patients [76]. This underestimation is mainly related to the intra-tumoural heterogeneity or presence of an insufficient amount of tumour in the core to allow 10 HPF to be counted for mitosis [77]. Although, hotspot usually found in the periphery of the tumour, where core biopsy is taken, in a previous study we found that a significant number of tumours had either scattered or central hotspot [74].

Ki67 and molecular subtypes

Ki67 and hormone receptor positive (luminal) BC: A strong negative correlation was found between hormone receptors and Ki67 levels [9 78-80]. Luminal tumours show a wide spectrum of proliferative activity, this reflected the ability of Ki67 to stratify hormone receptor positive BC into prognostically distinct groups based on the proliferative activity. The St Gallen Guidelines recommended Ki67 in order to distinguish the molecular subtypes luminal A and luminal B BC subtypes [11]. Due to its prognostic significance in hormone receptor positive BC, *MKi67* gene is one of the 16 genes that are measured in the Oncotype DX recurrence score (RS) [81-83]. There is a high concordance rate between Oncotype DX recurrence scores and Ki67 index [36 84 85]. In our recent study, we found a strong positive correlation between Oncotype DX RS and

Ki67 and none of the patients with less than 10% Ki67 expression had high risk Oncotype DX RS while in high Ki67 expression tumours, Oncotype DX RS varies. For example, 54% of tumours with Ki67 >70% has high risk RS compared to 17% that showed low risk RS, supporting the reliability of Ki67 in predicting tumour prognosis and Oncotype DX [83]. In addition, a previous study showed that high Ki67 tumours are at higher risk of relapsing among patients with low risk Oncotype DX, and Ki67 status may help to identify a subset of low risk Oncotype DX patients who could benefit from adjuvant chemotherapy [86]. Nottingham Px is another prognostic index, which incorporate Ki67 in addition to tumour grade, tumour size, and PR [87].

- **Ki67 and HER2 positive BC:** The correlation with HER2 status is less significant as HER2 positive and negative tumours show a wide range of proliferative activity. Some studies found positive correlation between Ki67 and HER2 [88 89] but others did not show any association [90]. This may also result from the difference of Ki67 cut-off points used. In some studies, the cut-off points of Ki67 positive were greater than 30% positive cells, while in others it was less than 12 or 25 % positive cells [91].
- **Ki67 and triple negative BC (TNBC):** The majority of hormone receptor negative BC are highly proliferative. A correlation is seen with high Ki67 index and TNBC [45] and high Ki67 expression is significantly associated with aggressive clinical behaviour in TNBC [92].

Stromal Ki67 expression in BC

Stromal expression of Ki67 could be helpful in the assessment of some cases including multinucleated giant stromal cells in benign fibroepithelial lesions, which show low Ki67 expression; however, in malignant giant cells, it showed high Ki67 expression [72]. In addition, expression of ki67 in stromal cells could be helpful in grading phyllodes tumours rather than mitotic figures that are affected by fixation issues [93].

Predictive role of Ki67

Ki67 can potentially serve as a tool to identify BC patients who could benefit from chemotherapy [94]. Previous studies demonstrated that that Ki67 is a marker of chemosensitivity in BC as most cytotoxic agents require cells to be in the cell cycle [42]. High proliferating tumours are associated with high risk of recurrence and poorer

outcomes, however, they are likely to response better to cytotoxic chemotherapy [95]. Ki67 has been shown to be an important part of a prognostic algorithm for residual risk in early BC patients treated with letrozole or tamoxifen [96].

The role of Ki67 in determining the eligibility for Neoadjuvant chemotherapy (NAC) has been tested. Two studies have showed that high Ki67 is associated with a good response to NAC [97 98]. It has been observed that patients with high post-treatment Ki67 levels are at higher risk for disease relapse and death compared with patients with low or intermediate Ki67 levels. Moreover, post-treatment Ki67 levels provided more prognostic information than pre-treatment Ki67 levels or changes of its levels from pre- to post-treatment. [97]. Importantly, a meta-analysis of 36 studies showed that Ki67 might be an independent predictor for pathologic complete response [99]. However, not all studies agreed with that [100 101].

Ki67 and artificial intelligence

The traditional scoring method of Ki67 staining by IHC, may be time-consuming, poorly reproducible for many pathologists, and liable to inter/intra-observer variability [102 103]. Fortunately, in the era of whole slide digital scanning technology, it is now possible to combine histopathological image analysis with artificial intelligence (AI) [104]. This may enable highly accurate and rapid workflows [105]. However, the question arises whether AI could be used to solve the problem of accurate Ki67 assessment on IHC stained sections. To date, the development of such automated approaches has been limited, including a lack of automated selection for of regions of interest or the hotspot areas [102]. Stalhammar and colleagues reported that automated Ki67 assessment methods are superior to manual assessment in terms of sensitivity and specificity and can improve inter-observer reproducibility of Ki67 assessment [106]. However, automated scoring methods has some defects in the identification of invasive tumour cells, especially in lymphocytic-rich tumours where some Ki67-positive lymphocytes may be identified as tumour cells leading to its overestimation [55]. To overcome this, a semi-automated evaluation method of Ki67 index which allows for the determination of the exact proliferation index value by marking tumour and non-tumour cells manually, followed by counting the cells automatically has been proposed [55]. Also, new software in development could overcome this problem through virtual double staining for tissue classification. For example, the same tissue section is stained for both cytokeratin and

Ki67; tumour cells are recognised by positive cytokeratin expression, and only cells that co-express both markers are automatically counted as positive tumour cells [106 107]. Efforts should be exerted to develop a standard methodology and recognised constant cut-offs with the development of computer-assisted image analysis guidelines, which can improve the reproducibility of Ki67 assessment [108].

Future direction and recommendation

We believe that Ki67 should be used in the routine practice as a reliable prognostic and predictive marker in early-stage BC in addition to its role as representative surrogate of tumour proliferation. In addition, Ki67 is easily accessible and competitive marker that is affordable in pathology laboratories.

We recommend using MIB1 antibody for Ki67 staining. Full face section is the optimal type of tissue sections for proper Ki67 scoring and for avoiding the under estimation of actual Ki67 value due to the effect of intra-tumoural heterogeneity of Ki67. Using a standardised method in Ki67 scoring, which depends on calibration of fields area of microscopes and visual estimation of Ki67 within 1000 cells without actual counting should be adopted. In terms of cut-off, we recommend the optimal cut-off for Ki67 categorization should be adjusted according to the context of Ki67 usage either for diagnostic, prognostic, or predictive value.

Conclusion

Ki67 is a valuable prognostic marker in early BC and represents a cost-effective approach to assess cellular proliferation compared to other methods. An accurate analysis of Ki67 will depend on consistent, reproducible, and valid scores in large cohorts. Ki67 can be simply assessed in routine practice by visually estimation of the average percentage of Ki67 positive cells in the area that contain approximately 1000 cells or more without counting the cells by calibrating the microscope HPF and tumour cellularity. Efforts should be exerted to develop a standard methodology and accepted cut-off that will enhance pathologists to properly assess this marker.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Ethics Approval

This study was approved by the Yorkshire & the Humber - Leeds East Research Ethics Committee (REC Reference: 19/YH/0293) under the IRAS Project ID: 266925. Data collected were fully anonymized.

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Figure legends

Figure 1: Representative images of different examples of positive Ki67 mitotic cells. Typical mitotic cells with Ki67 also stained in the areas around the chromosomes (perichromosomal layer) (a), atypical mitoses (b).

Figure 2: Representative expression of Ki67 in BC. Very low Ki67 expression (a), moderate Ki67 expression (b&c), high Ki67 expression (d&e) and very high ki67 expression in BC cells (f).

Figure 3: Patterns of Ki67 staining: a, homogenous staining of the nucleus. b, granular pattern granules of distinct size dispersed throughout the nucleoplasm and c, mixed pattern of homogenous and granular ones.

Table1: Comparison between Ki67 and other proliferative markers in breast cancer (BC)

Marker	Function	Cell cycle	Prognostic role in BC	Disadvantages	Correlation with ki67	Correlation with clinicopathological of BC
Ki67	Required during interphase for normal cellular distribution of heterochromatin antigens.	Expressed in all cell cycle phases except in G0.	Correlation with poor disease-free survival (DFS), overall survival (OS) and BC-specific survival (BCSS).	No standard scoring protocol or accepted cut-off point until now.		Correlate positively with histologic grade, Nottingham Prognostic Index (NPI), lymph node stage and HER2. Correlate inversely with ER and PR status. A good biomarker for selection the systemic treatment of patients with early-stage BC
PCNA	*Involved in DNA excision repair, cell cycle control, chromatin assembly, and RNA transcription*	Expressed in S and G phases.	Association with a shorter DFS and OS.	Not specific for proliferation as it corresponds with the cells in DNA replication sites.	Poor correlation.	Poor correlation with clinical parameters.

PHH3	Involved in chromatin condensation	Expressed in M phase only.	A prognostic marker in patients with lymph node-negative BC. Correlation with short DFS.	There is a debate concerning cut-off values assessment. Expression affected with fixation time.	Poor correlation.	Perfect correlation with mitotic index and grade. No correlation was found with hormonal receptors.
MCM family	Essential to ensure eukaryotic DNA is replicated only once per cell cycle.	Increased transcription during the G1/S phases.	Association with OS, BCSS, regional recurrence, and distant metastases free survival (DMFS).	Replication-competent when compared to Ki67. MCM is not superior to Ki67 in BC	Strong correlation. Higher expression in BC compared to Ki67.	Good correlation with clinicopathological parameters except lymph node stage and vascular invasion.
Topoisomerase II	Key enzyme for controlling of topological states of DNA	Expressed S/G2/M phases.	Association with a shorter DFS and OS.	Not exclusively expressed by proliferating cells but also during DNA damage repair.	Strong correlation.	Perfect correlation with high tumour grade and Ki67 index,