Assessment of proliferation in breast cancer: cell cycle or mitosis? An observational study

Ayat G Lashen,1,2 Michael S Toss,1 Ayaka Katayama,3 Rajan Gogna,1 Nigel P Mongan1,4 & Emad A Rakha1

1Division of Cancer and Stem Cells, School of Medicine, University of Nottingham, Nottingham, UK, 2Department of Pathology, Faculty of Medicine, Menoufia University, Shebin El Kom, Egypt, 3Diagnostic Pathology, Gunma University Graduate School of Medicine, Maebaashi, Japan, and 4School of Veterinary Medicine and Sciences, University of Nottingham, Nottingham, UK

Date of submission 7 May 2021
Accepted for publication 15 August 2021
Published online Article Accepted 28 August 2021

Assessment of proliferation in breast cancer: cell cycle or mitosis? An observational study

Background and aims: Proliferation is an important indicator of breast cancer (BC) prognosis, but is assessed using different approaches. Not all cells in the cell cycle are committed to division. This study aimed to characterise quantitative differences between BC cells in the cell cycle and those in mitosis and assess their relationship with other pathological parameters.

Methods and results: A cohort of BC sections (n = 621) was stained with haematoxylin and eosin and immunohistochemistry for Ki-67. The proportion of mitotic cells and Ki-67-positive cells was assessed in the same areas. The Cancer Genome Atlas (TCGA) BC cohort was used to assess MKI-67 transcriptome level and its association with the mitotic counts. The mean proportion of BC cells in the cell cycle was 24% (range = 1–90%), while the mean proportion of BC cells in mitosis was 5% (range = 0–73%). A low proportion of mitoses to whole cycling cells was associated with low histological grade tumours and the luminal A molecular subtype, while tumours with a high proportion of mitoses to the overall cycling cells were associated with triple-negative subtype, larger tumour size, grade 3 tumours and lymph node metastasis. The high mitosis/low Ki-67-positive cells tumours showed a significant association with variables of poor prognosis, including high-grade and triple-negative subtypes.

Conclusion: The proportion of BC cells in the cell cycle and mitosis is variable. We show that not only the number of cells in the cell cycle or mitosis, but also the difference between them, provides valuable information on tumour aggressiveness.

Keywords: breast cancer, cell cycle, Ki-67, mitosis, molecular classes, proliferation

Introduction

Tumour cell proliferative activity is an important independent prognostic and treatment predictive factor in breast cancer (BC) patients.1–3 There are multiple approaches to evaluate the proliferative activity of BC, but the most commonly used methods involve assessment of cells in the mitotic phase (mitotic counts, which is a component of the Nottingham histological grade) or quantifying the number of cells in the cell cycle, which is frequently evaluated using Ki-67 immunohistochemistry.1,3 Ki-67 is a labile non-histone protein, involved in the early steps
of polymerase I-dependent ribosomal RNA synthesis, and is present in all proliferating cells spanning the whole cell cycle from G1 to M phases. Although Ki-67 expression fluctuates during the cell cycle, with expression increasing to its maximal level during early mitosis and decreasing in later anaphase and telophase mitotic phases, its expression remains detectable during the various phases of the cell cycle, including the mitotic phase, and is absent in G0. Importantly, it has been shown that high levels of Ki-67 are associated with worse prognoses in BC, and has the advantage of identifying the proliferative cells outside mitosis.

Assessment of mitotic activity, which is a well-established and swift approach of evaluation of cell proliferation in BC, is based upon visual quantification of mitotic figures in haematoxylin and eosin (H&E)-stained slides. Mitotic counts, when assessed in well-fixed tissue, can produce equivalent prognostic information to Ki-67. Even though Ki-67 and mitotic counts in BC are used to assess the proliferative activity of BC, they do not necessarily identify the same cells. Not all cells in the cell cycle will proceed to the mitosis phase and become committed to cell division. Ki-67 is often used to quantify the percentage of positive BC cells, whereas mitotic counts represent the number of mitotic figures in a certain tumour area, referred to as a ‘hot-spot’. However, the frequency of BC cells in the cell cycle and mitosis and the relation between them remain to be characterised. This is particularly important considering the high degree of intratumoral heterogeneity in BC, which is associated with treatment resistance, and metastatic potential.

In this study we have quantified BC cells which were progressing in the cell cycle, as demonstrated by Ki-67 staining and those committed to cell division, as demonstrated by Ki-67-positive mitotic counts. The different proliferative activity of BC was assessed in the same area and the relationship of these parameters to clinicopathological variables was also assessed.

Material and methods

In this study, 621 patients with primary invasive BC, who received surgery without neoadjuvant treatment at Nottingham City Hospital, Nottingham, UK between 1999 and 2006, were enrolled and informed consent obtained. This is a well-characterised cohort with standardised tissue fixation protocols.

Clinicopathological data, including molecular subtypes, histological tumour type, primary tumour histological grade, lymph node status, lymph vascular invasion, Nottingham prognostic index (NPI) and tumour size were available (Table 1). The median tumour size was 1.9 cm (range = 0.2–10 cm), while the median NPI was 4.3 (range = 2.1–7.2). Information regarding oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) status was derived from the original pathology reports and assessed as per UK guidelines. For ER and PR, tumours were classified as...

| Table 1. The demographic characteristics of the study cohort |
|-------------------------------------------------------------|
| Categories | Number | % |
|------------|--------|---|
| **Molecular subtype**                        |        |   |
| Luminal A  | 148    | 23.8% |
| Luminal B  | 223    | 35.9% |
| Triple negative/Basal | 193 | 31.1% |
| HER2 enriched | 57 | 9.2% |
| **Histologic types**                          |        |   |
| No special type (NST) | 427 | 68.8% |
| Lobular    | 37     | 5.9%  |
| Other special types | 25 | 4.0%  |
| Mixed tumours | 132 | 21.3% |
| **Tumour grade**                             |        |   |
| Grade 1    | 74     | 11.9% |
| Grade 2    | 200    | 32.2% |
| Grade 3    | 347    | 55.9% |
| **Lymph node status**                        |        |   |
| Negative   | 410    | 66.0% |
| Positive   | 211    | 34.0% |
| **Lymph vascular invasion**                  |        |   |
| Absent     | 419    | 67.5% |
| Present    | 202    | 32.5% |
| **Nottingham prognostic index (NPI)**         |        |   |
| Good prognostic group                        | 154    | 24.8% |
| Moderate prognostic group                    | 364    | 58.6% |
| Poor prognostic group                        | 103    | 16.6% |

© 2021 John Wiley & Sons Ltd, Histopathology, 79, 1087–1098.
positive when there was ≥1% nuclear staining in invasive carcinoma cells. HER2 immunohistochemistry (IHC) was classified as positive (3+), equivocal (2+) or negative (1+/0). Patients with 2+ HER2 IHC were tested for HER2 amplification by fluorescence in-situ hybridisation (FISH). HER2 IHC scores of 3+ or 2+ with a ratio ≥2.0, regardless of the HER2 copy number or where HER2 copy number was ≥6, were considered HER2-positive, consistent with UK guidelines. Other histological information used in this study was reviewed and evaluated by at least two pathologists to select the representative section, which contains the highest tumour burden.

Tumours were classified into four histological types: (i) invasive breast carcinoma, no special type (NST), (ii) lobular carcinoma, (iii) other special types and (iv) mixed NST and special types (this group was defined based on the 2019 WHO 5th edition, as NST admixed with special type comprises 11–90% of the tumour). Tumours were also classified based on ER, PR and HER2 into three molecular subtypes (luminal, triple-negative, HER2-enriched). According to the IHC expression of Ki-67 in the hot-spot, luminal cases were divided into two subtypes, luminal A and luminal B, based on the Ki-67 14% cut-off.

Full-face sections were prepared from the selected formalin-fixed, paraffin-embedded (FFPE) blocks per case. For mitotic counts, H&E staining was performed on FFPE tissue. For Ki-67, FFPE tissue sections were heated for 10 min on a hot plate at 60°C and allowed to cool. The sections were deparaffinised, rehydrated and antigen retrieval achieved by microwaving in citrate buffer (pH 6.0). Immunohistochemistry was performed with peroxidase and protein blocking, each for 5 min, followed by primary antibody (anti-human Ki-67 monoclonal antibody MIB1; Dako, Glostrup, Denmark) incubation for 3 min. Counterstaining was performed using Meyer’s haematoxylin for 6 min and the sections were dehydrated and then coverslipped using dibutyl phthalate in xylene (DPX). Positive control of normal tonsil was included for each staining run, while negative control was used by omitting the primary antibody incubation from the staining protocol. The immunohistochemical staining was performed automatically using the Dako Cytomation EnVision+ detection system.

**Mitosis counting**

Quantification of mitoses was performed at ×400 magnification using a light microscope (Eclipse Ni-U; Nikon Instruments Inc., Tokyo, Japan) using ×10 ocular lens (eyepieces) with field-of-view (FOV) of 25 mm and ×40 objective lens with field diameter of 0.63 mm. Hot-spot areas were selected based on being the most cellular area, and were most commonly located at the peripheral invasive front of the tumour. The fields with the highest mitotic counts were selected to commence the counts. Only cells with clear mitotic morphology were counted as defined by (i) absence of the nuclear membrane, (ii) clearly visible hairy extension of nuclear material (condensed chromosome), (iii) either clotted (beginning metaphase), in plane (metaphase/anaphase) or separate clots (telophase). Supporting information, Figure S1 shows example illustrations for these figures. Ambiguous cells with a hyperchromatic nucleus or cells suspected of apoptosis were excluded.

**Ki-67 scoring (eyeballing visual assessment)**

Ki-67 was assessed by three methods as follows: (1) Ki-67 in hot-spots, (2) the average Ki-67-positive tumour cells and (3) Ki-67-positive mitotic cells. The details of the three methods were as follows.

1. **Ki-67 in the hot-spots:** these were quantified by examining stained sections using a standard light microscope with a ×40 objective. Only nuclear staining was counted, and the Ki-67 score was defined as the percentage of the total number of tumour cells in 1000 tumour cells in the hot-spot area, defined as the areas with the highest density of Ki-67-positive tumour cells compared to the surrounding tumour tissue areas.

2. **Average Ki-67:** we calculated the average Ki-67 score using two methods: (i) the number of Ki-67 nuclear staining cells in 1000 cells in multiple randomly selected areas regardless of the location of the hot-spot and (ii) the percentage of Ki-67-positive cells in the whole tumour area (calculated by scanning the entire slide at low power magnification (×100). All areas of tumour-positive expression were estimated including areas of highest and lowest positivity and the average percentage of expression was assessed.

3. **Counting Ki-67-positive mitotic cells:** this was assessed by calculating the number of Ki-67-positive stained mitotic figures in 1000 cells in hot-spot areas. To also assess the ability of Ki-67 to stain mitosis effectively, we quantified Ki-67-negative mitotic cells in 1000 malignant cells in the same area.

The relationship between mitosis and Ki-67 was assessed by comparing mitotic scores in H&E against Ki-67 (hot-spot and average). It is important to note that,
in our study. Ki-67 was scored blinded to the manual mitotic count for each case. In addition, Ki-67 was confirmed by evaluation by another pathologist who independently scored 50% of the cohort (n = 303). The interclass correlation coefficient (ICC) between two independent assessors for Ki-67 in hot-spots and the average and for assessment of Ki-67-positive mitotic cells were 0.9, 0.8 and 0.7, respectively.

**EVALUATION OF THE PROPORTION OF BC CELLS IN THE CELL CYCLE ENTERING MITOSIS**

To evaluate the proportion of BC cells in the cell cycle entering mitosis, we counted Ki-67-positive mitotic cells in 1000 cells in a hot-spot then converted this number into a percentage (percentage of mitosis in 1000 cells = number of mitoses per 1000 cells/1000 × 100). Consequently, if we consider that Ki-67 represents the total number of BC cells in the cell cycle, the proportion of cells entering mitosis is equal to the percentage of Ki-67-positive mitotic cells in 1000 cells/percentage of Ki-67-positive in 1000 cells × 100.

For statistical purposes, absolute mitotic counts were converted into scores 1, 2 and 3 using existing cut-off points, according to the College of American Pathologists (CAP) recommendations, where 0–11 mitoses per 10 high-power field (HPF) is equivalent to score 1 and 12–22 mitoses for score 2, while score 3 is considered when >22 mitoses per 10 HPF.31,32 To define the different proliferative activities of BC cells, we have categorised the whole cohort into four groups based on Ki-67-positive mitotic counts and Ki-67 in hot-spots: group 1 = low mitosis, low Ki-67; group 2 = high mitosis, high Ki-67; group 3 = low mitosis, high Ki-67; and group 4 = high mitosis, low Ki-67 (Supporting information, Figure S2). Furthermore, the relationship between these groups and available clinicopathological parameters was assessed. In addition, we assessed the relationship between the hot-spot for Ki-67 and the hot-spot for mitosis.

**THE TRANSCRIPTOMIC COHORT**

In addition, digital H&E BC images from The Cancer Genome Atlas (TCGA)27 (n = 388) were used to compare mitotic counts to the tumour MKI-67 mRNA expression level, which represents the whole tumour tissue proliferative activity. The full clinicopathological data and MKI-67 mRNA expression were available for this cohort. Whole slide images were downloaded from the cBioPortal website and viewed on CaseViewer software (version 2.2.0.85; 3D-Histech, Budapest, Hungary). Mitotic figures were scored on the H&E images by one observer (A.L.). Mitotic counts and MKI-67 were categorised, based on the median, into low and high groups. To confirm the dynamic relationship between mitosis and Ki-67, the whole cohort was categorised into four proliferative groups: group 1 = low mitosis, low MKI-67; group 2 = high mitosis, high MKI-67; group 3 = low mitosis, high MKI-67; and group 4 = high mitosis, low MKI-67. Their relationship with the available clinicopathological parameters was also evaluated.

**STATISTICAL ANALYSIS**

The Statistical Package for the Social Sciences software version 26.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Spearman’s rank correlation was used to correlate for continuous variables. The percentage of positive Ki-67 cells was also categorised as low (<10%), intermediate (10–20%) or high (>20%).33 The χ² test was used for analysis of categorical 2 × 2 data while R software (R version 4.1.0; Camp Pontanezen, Brest, France) was used for analysis of more than two categorical variables. The Mann–Whitney test was used for assessing non-parametric data. The degree of interobserver agreement was assessed by the interclass correlation coefficient (ICC) for continuous data. For statistical analysis, Ki-67-positive mitotic counts were categorised based on the median. The median of mitoses counts was 10 mitoses, which stratified the cohort into low (≤10) and high (>11) groups. Similarly, Ki-67-positive cells were categorised into low and high groups based on the median (21%). The proportion of BC cells in the cell cycle entering mitosis was categorised based on the median, into low and high groups, and was correlated with the available clinicopathological parameters. A P-value ≤0.05 was considered statistically significant.

This study was approved by the Yorkshire and The Humber–Leeds East Research Ethics Committee (REC reference: 19/YH/0293) under the IRAS Project ID: 266925. Data collected were fully anonymised.

**Results**

The mean ± standard deviation (SD) of cycling, i.e. Ki-67-positive cells, in the whole tumour slide were 24 ± 22% and the median was 17% (<1–95%); the mean ± SD in the hot-spots were 31 ± 27% and the median was 21% (range=1–100%).

Of the 621 informative cases assessed 50% showed high proliferative activity, with a high proportion of cells...
in the cell cycle when hot-spots were assessed. However, when using the average method (in the whole slide) this proportion dropped to 40%. Table 2 shows the distribution of cycling cells and the cells in mitosis.

There was a strong association between cycling cells and cells in the mitosis regardless of the area of assessment (in hot-spots or the average) ($P < 0.0001$), with an overlap of mitotic counts and Ki-67-positive cells observed in 90% of cases (Table 3, Figures 1 and 2). The mean ± SD of Ki-67-positive mitotic cells was 13 ± 12 mitoses (range = 0–60 mitoses) (Figures 3 and 4).

**Table 2. Distribution of Ki-67 and mitotic scores in the study cohort**

| Categories                      | Number | %    |
|---------------------------------|--------|------|
| **Ki-67 expression in hotspot** |        |      |
| Low                             | 170    | 27.4%|
| Intermediate                    | 138    | 22.2%|
| High                            | 313    | 50.4%|
| **Ki-67 expression in average** |        |      |
| Low                             | 218    | 35.1%|
| Intermediate                    | 157    | 25.3%|
| High                            | 246    | 39.6%|
| **Ki-67-positive mitotic cells**|        |      |
| Low                             | 316    | 50.9%|
| High                            | 305    | 49.1%|
| **Mitotic counts in H&E**       |        |      |
| Score 1                         | 264    | 42.5%|
| Score 2                         | 123    | 19.8%|
| Score 3                         | 234    | 37.7%|

Ki-67-positive cells: low (<10%), intermediate (10 to 20%); high (>20%). Mitotic scores (score 1: 0–11 mitoses; score 2: 12–22 mitoses; score 3: >22 mitoses). Ki-67 positive mitotic cells: low (≤10 mitoses), high (>11 mitoses).

The whole cohort was stratified into four proliferative groups based on Ki-67-positive mitotic counts and Ki-67-positive cells in 1000 cells in hot-spots using the same area. This demonstrated that 42% of cases (260 of 621) show low proliferative activity and low mitotic activity (low mitosis/low Ki-67), while 40% (251 of 621) had high mitosis/high Ki-67. In contrast 18% (110 of 621) showed discrepant results; 10% showed low mitosis/high Ki-67 and 8% showed high mitosis/low Ki-67.

The first group (low mitosis/low Ki-67) showed a significant association with luminal A molecular subtypes, lobular carcinoma type, lymph node negativity, small tumour size and grade 1 tumours ($P < 0.001$). The second group (high mitosis/high Ki-67) showed a significant association with grade 3 tumours, as 66% of grade 3 tumours showed high mitosis and high Ki-67 ($P < 0.0001$).

Patients with discordant cell cycle and mitosis counts were assessed as separate groups and correlated with their corresponding clinicopathological parameters. The low mitosis with high Ki-67 group

© 2021 John Wiley & Sons Ltd, Histopathology, 79, 1087–1098.
showed a significant association with the luminal B subtype ($P < 0.0001$). In contrast, the high mitosis with low Ki-67 group was significantly associated with triple-negative subtype, grade 3 tumours and higher NPI ($P < 0.0001$). Table 5 shows the relationship between the discrepancy groups and the clinicopathological parameters.

**THE TRANSCRIPTOMIC COHORT**

BC cases ($n = 388$) from TCGA external cohort were used to determine the proportion of BC in the cell cycle. In those cases, the mean ± SD mitotic counts were $13 ± 11.5$, the median was 10 (range = 0–54), the mean ± SD MKi-67 was $2333 ± 1883$ and the transcripts and median were 1712.7 transcripts.

Of these 388 patients, 51% (196 of 388) showed low proliferative activity (low MKi-67 expression), while 49% (192 of 388) had high proliferative activity (high MKi-67 expression). Similar to IHC, there was a significant association between MKi-67 expression and mitosis ($P < 0.0001$).

To confirm the different proliferative activity of BC cells, this cohort was categorised into four proliferative groups based on H&E mitotic counts and mKi-67 expression levels; 37% of the cases showed low

---

**Table 3. Relationship between mitotic scores and scores of cycling (Ki-67-positive) cell scores (low, intermediate, high)**

| Variables        | Mitosis scores | Proportion of cycling cells | 1 Number (%) | 2 Number (%) | 3 Number (%) | $P$-value |
|------------------|----------------|----------------------------|--------------|--------------|--------------|-----------|
| In hotspots      |                |                            |              |              |              |           |
| Low              |                | 133 (78.2%)                | 19 (11.2%)   | 18 (10.6%)   |              | $<0.0001$ |
| Intermediate     |                | 80 (58.0%)                 | 35 (25.3%)   | 23 (16.7%)   |              |           |
| High             |                | 51 (16.3%)                 | 69 (22%)     | 193 (61.7%)  |              |           |
| Average          |                |                            |              |              |              |           |
| Low              |                | 17 (78.4%)                 | 25 (11.5%)   | 22 (10.1%)   |              | $<0.0001$ |
| Intermediate     |                | 67 (42.7%)                 | 50 (31.8%)   | 37 (25.5%)   |              |           |
| High             |                | 26 (10.6%)                 | 48 (19.5%)   | 172 (69.9%)  |              |           |

Ki-67-positive cells: low (<10%), intermediate (10 to 20%); high (>20%). Mitotic scores (in H&E and in Ki67per 10 HPF): (score 1: 0–11 mitoses; score 2: 12–22 mitoses; score 3: >22 mitoses).

Significance $P$ values are in bold.

**Figure 1.** Box-plot shows the relationship between mitotic scores in haematoxylin and eosin- and Ki-67-positive cells in the hot-spot. [Colour figure can be viewed at wileyonlinelibrary.com]
mitosis/low MKI-67, while 34% showed high mitosis/high MKI-67. Conversely, 29% showed discrepancy groups, 15% had low mitosis/high MKI-67 and 14% showed high mitosis/low MKI-67.

Similar to the IHC groups, there was a significant association between the first group (low mitosis/low MKI-67) and the lobular carcinoma type and the luminal molecular subtype. The second group (high mitosis/high MKI-67) showed a significant association with NST histological type and triple-negative subtype. Also, there was a significant association with grade 3 tumours, the presence of necrosis and larger tumour size ($P < 0.0001$).

**Discussion**

Assessment of the proliferative activity of BC is an important tool to augment traditional clinicopathological parameters obtained during routine
histopathological examination of resected BC specimens.34 Both Ki-67, which stains all the cells in the cell cycle, and mitotic scores, which count the number of cells in mitosis and are committed to cell division, are used interchangeably.35–37 Although Ki-67 IHC has attracted much attention as a measure of the proliferative activity of BC, assessment of mitotic activity is a well-established approach, which is carried out in H&E-stained sections and is part of the Nottingham grading system.17 Although not all cycling tumour cells will progress to the mitotic phase and divide, there is concern that the mitosis represents the smallest proportion of cycling cells, thus not precisely reflecting the proliferation rate of tumours.38,39

Although various studies have demonstrated that mitotic counts and Ki-67 expression are positively

Figure 4. Matched mitotic figures between haematoxylin and eosin and Ki-67. A, Prophase shows a fine hairy-like projection, positive for Ki-67. B, Prophase shows a slightly irregular outline, positive for Ki-67. C, Metaphase shows a fine hairy projection and a slightly irregular outline, positive for Ki-67. D, Lag atypical mitosis shows an unattached chromatid to metaphase, hairy outline, positive for Ki-67. [Colour figure can be viewed at wileyonlinelibrary.com]

Figure 5. Box-plot shows the relationship between the proportion of breast cancer cells in the cell cycle entering mitosis and molecular subtypes. [Colour figure can be viewed at wileyonlinelibrary.com]
correlated, the dynamic relationship between them is not fully explored. In addition, the characterisation of BC in the cell cycle remains poorly defined. In the current study, we quantified the proportion of BC cells in the cell cycle and in mitosis using full face sections of excision specimens and both markers were assessed in the same areas using well-defined methodology. We have categorised the cohort into four groups based on Ki-67-positive cells and Ki-67-positive mitotic counts using the same tissue area to evaluate the agreement between these two measures to more clearly define the proliferative activity of BC cells. In view of the presumed subjectivity of scoring

### Table 4. Relationship between proportions of cycling breast cancer cells entering mitotic phase and clinicopathological diameters

| Categories                  | Low mitosis proportion Number (%) | High mitosis proportion Number (%) | P-value |
|-----------------------------|-----------------------------------|------------------------------------|---------|
| Molecular subtype           |                                   |                                    |         |
| Luminal A                   | 102 (68.9%)                       | 46 (31.1%)                         | <0.0001 |
| Luminal B                   | 123 (55.2%)                       | 100 (44.8%)                        |         |
| Triple negative/basal       | 64 (33.2%)                        | 129 (66.8%)                        |         |
| HER2 enriched               | 21 (36.8%)                        | 36 (63.2%)                         |         |
| Histologic types            |                                   |                                    |         |
| NST no special type         | 184 (43.1%)                       | 243 (56.9%)                        | <0.0001 |
| Lobular                     | 26 (70.3%)                        | 11 (29.7%)                         |         |
| Other special types         | 20 (80%)                          | 5 (20%)                            |         |
| Mixed tumours               | 80 (60.6%)                        | 52 (39.4%)                         |         |
| Tumour grade                |                                   |                                    |         |
| Grade 1                     | 60 (81.1%)                        | 14 (18.9%)                         | <0.0001 |
| Grade 2                     | 137 (68.5%)                       | 63 (31.5%)                         |         |
| Grade 3                     | 113 (32.6%)                       | 234 (67.4%)                        |         |
| Lymph node status           |                                   |                                    |         |
| Negative                    | 218 (53.2%)                       | 192 (46.8%)                        | 0.02    |
| Positive                    | 92 (43.6%)                        | 119 (56.4%)                        |         |
| Lymphovascular invasion     |                                   |                                    |         |
| Absent                      | 226 (53.9%)                       | 193 (46.1%)                        | 0.004   |
| Present                     | 84 (41.6%)                        | 118 (58.4%)                        |         |
| NPI                         |                                   |                                    |         |
| Good prognostic group       | 115 (74.7%)                       | 39 (25.3%)                         | <0.0001 |
| Moderate prognostic group   | 168 (46.2%)                       | 196 (53.8%)                        |         |
| Poor prognostic group       | 27 (26.2%)                        | 76 (73.8%)                         |         |

Significance P values are in bold.

NST, no special type; NPI, Nottingham prognostic index.

### Table 5. Relationship between the discrepant mitosis/Ki-67 groups and clinicopathologic parameters

| Categories                  | Low mitosis/ high Ki67 Number (%) | High mitosis/ low Ki67 Number (%) | P-value |
|-----------------------------|-----------------------------------|-----------------------------------|---------|
| Molecular subtype           |                                   |                                    |         |
| Luminal A                   | 0 (0.0%)                          | 1 (100.0%)                        | <0.0001 |
| Luminal B                   | 50 (78.1%)                        | 14 (21.9%)                        |         |
| Triple negative/basal       | 9 (25.7%)                         | 26 (74.3%)                        |         |
| HER2 enriched               | 4 (40.0%)                         | 6 (60.0%)                         |         |
| Histologic types            |                                   |                                    |         |
| NST                          | 45 (53.6%)                        | 39 (46.4%)                        | 0.3     |
| Lobular                     | 3 (75.0%)                         | 1 (25.0%)                         |         |
| Other special types         | 0 (0.0%)                          | 1 (100.0%)                        |         |
| Mixed tumours               | 15 (71.4%)                        | 6 (28.6%)                         |         |
| Tumour grade                |                                   |                                    |         |
| Grade 1                     | 3 (100.0%)                        | 0 (0.0%)                          | <0.0001 |
| Grade 2                     | 35 (85.4%)                        | 6 (14.6%)                         |         |
| Grade 3                     | 25 (37.9%)                        | 41 (62.1%)                        |         |
| Lymph node status           |                                   |                                    |         |
| Negative                    | 39 (61.9%)                        | 24 (38.1%)                        | 0.3     |
| Positive                    | 24 (51.1%)                        | 23 (48.9%)                        |         |
| Lymphovascular invasion     |                                   |                                    |         |
| Absent                      | 40 (57.1%)                        | 30 (42.9%)                        | 0.9     |
| Present                     | 23 (57.5%)                        | 17 (42.5%)                        |         |
| NPI                         |                                   |                                    |         |
| Good prognostic group       | 15 (88.2%)                        | 2 (11.8%)                         | <0.0001 |
| Moderate prognostic group   | 41 (57.7%)                        | 30 (42.3%)                        |         |
| Poor prognostic group       | 7 (31.8%)                         | 15 (68.2%)                        |         |

Significant P values are in bold.

NST, no special type; SD, standard deviation; NPI, Nottingham prognostic index.
of Ki-67, we started this study by assessment of 50% of this cohort by two pathologists and then calculated the concordance rate between them, which showed almost perfect agreement. Therefore, we decided to continue scoring by one pathologist. Although the mitotic count was carried out by one observer, we have compared the scoring with the available data obtained from the original mitotic score assigned for the cases at the time of clinical reporting, which showed a kappa value of 0.8.

Our results showed that a strong association between the proportion of cycling cells and cells in mitosis and the mean percentage of cycling cells (Ki-67 positive cells) in BC ranged from 24 to 30% based on the method of assessment, whether in the whole area or the hot-spots. A small majority (52%) of BC showed low proliferative activity (low Ki-67-positive cells), whereas 48% of cases showed high proliferation. To the best of our knowledge, this study is the first to quantify the number of Ki-67-positive and negative mitotic figures (Figures 3 and 4), and we have used this approach to accurately assess the percentage of BC cells entering mitosis to complement mitotic figure assessment. To do this, we assessed the proportion of Ki-67-positive mitotic figures compared to all Ki-67-positive cells in the same area. We found that the mean proportion of BC cells in mitosis was ~5%, range = 0–73%. Interestingly, a high proportion of cycling BC cells in mitosis was significantly associated with features of aggressive clinical phenotypes, including the triple-negative subtype, grade 3 tumours ($P < 0.0001$), larger tumour size ($P < 0.003$) and the presence of lymph node metastasis and LVI. This suggests that quantification of cycling BC cells in mitosis may be useful to predict tumour aggressiveness. This is supported by recent evidence showing that the entry of cancer cells into mitosis sensitisises tumour cells to new anti-cancer strategies. Similarly, Lee and colleagues reported that the proportion of cells entering mitosis might be calculated from the mitotic counts compared to the Ki-67 expression. Furthermore, we have explored the correlation between mitosis and Ki-67 hot-spots in heterogeneous tumours and 90% (135 of 150) exhibited overlapping of Ki-67 and mitosis within and adjacent to hot-spots. This indicates that the same hot-spots of both markers may reflect the most biologically active part of the tumour, which drives the outcome of the disease.

To define the different proliferative activity of BC cells, the whole cohort was categorised into four proliferative groups according to Ki-67-positive cells and Ki-67-positive mitotic figures in 1000 cells within a hot-spot. We found that 42% of the cohort harboured both low mitosis and low Ki-67, and this was significantly associated with luminal A molecular subtypes, lobular carcinoma, grade 1 tumours, absence of lymph node invasion, negative LVI, small tumour size and low NPI. This suggests that the low Ki-67 and low mitosis groups may more clearly distinguish tumours with better outcomes. In contrast, 40% of this cohort possessed both high mitosis and high Ki-67, which was significantly associated with high-grade tumours. Also, 50% of NST tumour type had high mitosis with high Ki-67.

Notably, 18% of our cohort showed discrepant Ki-67 and mitosis, where 10% showed low mitosis/high Ki-67 and 8% showed high mitosis/low Ki-67. Furthermore, the low mitosis/high Ki-67 group was significantly associated with the luminal B subtype. The high Ki-67 expression in this group may give a false proliferation index, as this group did not show a significant association with any of the other poor clinicopathological parameters.

This group may result from pre-analytical factors, as mitosis seems to be more affected with poor fixation than Ki-67 immunohistochemistry. However, the Nottingham cohort is well fixed, as we follow strict fixation and processing protocol which is uniformly applied to all cases. We believe that the low mitosis/high Ki-67 group represents the group of BC with a high proportion of cells in the cell cycle, but only few of them entered mitosis. For example, some cells may stop at a certain point of the cycle, either stop their cycles or go into apoptosis. Additionally, it may be related to the length of S-phase duration, as it has been found that an increasing DNA content, as well as an increasing percentage of cells in S-phase, were both correlated with increasing Ki-67 expression.

The last group (high mitosis/low Ki-67) showed a significant association ($P < 0.001$) with triple-negative subtype, grade 3 tumours and higher NPI. This group was associated with poor prognosis and thus may require more aggressive therapy than the other proliferative groups. The explanation of this group seems to be difficult; in a previous study they showed that mitotic blocks for DNA repair may lead to a larger number of mitotic figures being counted. However, this could explain a small number of mitotic cells which do not reach 8% of the cohort, as we found in the current study. We believe that this BC group showed the highest proliferative activity, as most of the cycling cells were committed to cell division and hence were captured in the mitosis phase. Similarly, a previous study of discrepant Ki-67 and mitosis showed that high mitosis with low Ki-67

© 2021 John Wiley & Sons Ltd, Histopathology, 79, 1087–1098.
showed a similar poor outcome to high mitosis with high Ki-67.\textsuperscript{42} To further explore this group, we have compared the percentage of both Ki-67-positive cells and Ki-67-positive mitotic cells in the same area and found that the percentage of Ki-67-positive cells outweigh the percentage of Ki-67-positive mitotic cells, as Ki-67 stains all malignant cells entering cell cycles, including mitotic cells. The mean ratio between both groups (the percentage of Ki-67-positive cells to the percentage of Ki-67-positive mitotic cells was 16:1; range = 5:1–60:1). This explains that the Ki-67 percentage was falsely interpreted when compared to the absolute mitotic counts. Previously, these groups were classified based on mitotic counts in H&E-stained slides and Ki-67 expression,\textsuperscript{13} but we have assessed Ki-67 and mitosis in the same area on hotspots of Ki-67-stained sections for consistency. In the TCGA cohort we observed a statistically significant association ($P < 0.0001$) between mitosis scores and MKi-67 expression levels. Furthermore, 51% of such cases showed low proliferative activity. These findings were similar to our IHC results, as 52% of our patients showed low proliferation; 29% of these cases showed a discrepancy between mitosis and MKi-67 compared to 18% in our cohort. The lower discrepancy group in our cohort may reflect comparing mitosis and Ki-67 in the same area.

In addition, the low mitosis/low MKi-67 group was significantly associated with good prognostic parameters, while the high mitosis/high MKi-67 group showed a significant association with poor prognostic parameters. However, no significant association was found between the discrepancy groups and the clinicopathological parameters in such cases. Finally, we have quantified BC cells in the cell cycle and in mitosis and shown the difference between them, which could have prognostic value.

**Conclusion**

Assessment of the proportion of cycling breast cancer and cells committed to cell division in routine pathology practice by mitosis and Ki-67 could provide valuable information on tumour aggressiveness.

**Acknowledgements**

A.L. is supported by and funded by the Egyptian Ministry of Higher Education and Scientific Research. We thank the PathLAKE digital pathology consortium. These new centres are supported by a £50m investment from the Data to Early Diagnosis and Precision Medicine strand of the government’s Industrial Strategy Challenge Fund, managed and delivered by UK Research and Innovation (UKRI).

**Conflicts of interest**

The authors declare that they have no conflicts of interest.

**References**

1. van Diest PJ, van der Wall E, Baak JP. Prognostic value of proliferation in invasive breast cancer: a review. *J. Clin. Pathol.* 2004; 57: 675–681.
2. Leong AS, Zhuang Z. The changing role of pathology in breast cancer diagnosis and treatment. *Pathobiology* 2011; 78: 99–114.
3. Elmi A, McDonald ES, Mankoff D. Imaging tumor proliferation in breast cancer: current update on predictive imaging biomarkers. *PET Clin.* 2018; 13: 445–457.
4. Beresford MJ, Wilson GD, Makris A. Measuring proliferation in breast cancer: practicalities and applications. *Breast Cancer Res.* 2006; 8: 216.
5. Soliman NA, Yussif SM. Ki-67 as a prognostic marker according to breast cancer molecular subtype. *Cancer Biol. Med.* 2016; 13: 496–504.
6. Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J. Immunol.* 1984; 133: 1710–1715.
7. Miller I, Min M, Yang C et al. Ki67 is a graded rather than a binary marker of proliferation versus quiescence. *Cell Rep.* 2018; 24: 1105–1112.e1105.
8. Bullwinkel J, Baron-Lühr B, Lüdemann A, Wohlenberg C, Gerdes J, Scholzen T. Ki-67 protein is associated with ribosomal RNA transcription in quiescent and proliferating cells. *J. Cell. Physiol.* 2006; 206: 624–635.
9. Urruticoechea A, Smith IE, Dowsett M. Proliferation marker Ki-67 in early breast cancer: a meta-analysis of published studies involving 12 155 patients. *Br. J. Cancer* 2007; 96: 1504–1513.
10. Muftah AA, Aleskandaramy MA, Al-kaabi MM et al. Ki67 expression in invasive breast cancer: the use of tissue microarrays compared with whole tissue sections. *Breast Cancer Res. Treat.* 2017; 164: 341–348.
11. Agboola AOJ, Banjo AAF, Anunobi CC et al. Cell proliferation (Ki-67) expression is associated with poorer prognosis in nigerian compared to british breast cancer women. *ISRN Oncol.* 2013; 2013: 675051.
12. Rossi L, Laas E, Mallon P et al. Prognostic impact of discrepant ki67 and mitotic index on hormone receptor-positive, HER2-negative breast carcinoma. *Br J Cancer* 2015; 113: 986–1002.
13. Kim J-Y, Jeong HS, Chung T et al. The value of phosphohistone H3 as a proliferation marker for evaluating invasive breast cancers: a comparative study with Ki67. *Oncotarget* 2017; 8: 65064–65076.
