Distribution pattern of the Ki67 labelling index in breast cancer and its implications for choosing cut-off values

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Abstract

The Ki67 labelling index (LI – proportion of staining cells) is widely used to reflect proliferation in breast carcinomas. Several cut-off values have been suggested to distinguish between tumours with low and high proliferative activity. The aim of the current study was to evaluate the distribution of Ki67 LIs in breast carcinomas diagnosed at different institutions by different pathologists using the method reflecting their daily practice. Pathologists using Ki67 were asked to provide data (including the LI, type of the specimen, receptor status, grade) on 100 consecutively stained cases, as well as details of their evaluation. A full dataset of 1709 carcinomas was collected from 19 departments. The median Ki67 LI was 17% for all tumours and 14% for oestrogen receptor-positive and HER2-negative carcinomas. Tumours with higher mitotic counts were associated with higher Ki67 LIs. Ki67 LIs tended to cluster around values ending with 5 or 0 both in cases where the values were obtained by counting the proportion of stained tumour cell nuclei and those where the values were obtained by estimation. On the basis of the distribution pattern described, some currently used Ki67 LI cut off values are not realistic, and it is proposed to select more realistic values ending with 0 or 5.

Keywords: Ki67; Proliferation; Breast cancer

Ki67 is a proliferation marker protein, which is expressed through the cell cycle, except in the G0 phase [1]. Its expression can be easily visualized by immunohistochemistry (IHC). This is why it is widely used to reflect the prognosis of several malignancies, including breast cancer. For this, the labelling index (LI), i.e. the proportion of positive (immunostained) cells is taken into account. Some of the labelled cells undergo apoptosis, whereas others remain in one of the gaps (G1 or G2), and still others progress to mitosis, but the LI is generally accepted as a parameter reflecting proliferation, and prognosis.

The protein has a differential expression during the cell cycle peaking during the M phase, and being much lower during G1 and S phases [2]. This differential expression is one limiting factor in assessing the proportion of immunostained cells, as the intensity the evaluating pathologist or software will include as positive cell is variable. Technical aspects of prefixation ischemic time, tissue fixation, processing, immunostaining and counterstaining may also alter both the staining intensity and the fraction of stained cells. Tumour areas may show significant heterogeneity of proliferation and Ki67 labelling and while it is common to see higher proliferation rates at the periphery of breast cancers, even the periphery may have an uneven distribution of positive cells, or areas anywhere in the tumour may proliferate more than others, leading to the appearance of so called “hot spots”. There is no consensus whether Ki67 staining evaluation should only consider the hot spots if present, just include them in a general count or avoid them altogether. The number of cells to be counted for this evaluation is also subject to variability. There are recommendations to consider at least 1000 cells [3], [4] and [5], or several hundred cells, but often only 100 cells are taken into account for counting [6] or only an estimation is made on the basis of larger areas. Because of these aspects, the reproducibility of the Ki67 LI is less than optimal. Despite efforts to standardize the determination of the Ki67 proliferation index, surprisingly eyeballing based estimation resulted in the most reproducible results [7].

Despite these problems, Ki67 labelling has been evaluated in many facets of prognostic breast pathology, and even recommended for clinical decision making by some authors [8] and [9].

For example, tumours with Ki67 labelling below 16% have been considered of low proliferation (and low risk), while tumours with Ki67 labelling above 30% have been considered of high proliferation (and high risk) by the St Gallen Consensus Conference in 2009 [8]. These cut off values were based on the best match between the Ki67 index and standardized mitotic rate [10]. Another publication suggests that oestrogen receptor (ER)-positive tumours with a high proliferation (defined as >13% Ki67 LI) are likely to belong to the luminal B class of ER-positive and HER2-negative breast cancers with a sensitivity of 77% and a specificity of 78% based on a comparison of molecular typing and immunohistochemical typing in tissue microarrays [11]. Yet another example suggests that histological grade 2 tumours with Ki67 LI less than 10% would behave like grade 1 tumours whereas those with a higher LI are expected to have a prognosis closer to grade 3 tumours [3]. (It is interesting to note, that the illustration for the low proliferation group in the last publication depicts about 144 tumour cells of which about 49 stain positively for Ki67, suggesting a percentage of positive cells much higher than 10%, reflecting that evaluating just a fraction of the 1000 cells eventually evaluated in the given study may distort the results) [3]. On the basis of a meta-analysis of 35 studies on the overall survival based prognostic role of Ki67 the following cut-offs were used for distinguishing between high and low proliferating tumours in individual studies: 3.5% (n = 1), 5% (n = 1), 7% (n = 1), 7.5% (n = 1), 8% (n = 1), 9% (n = 1), 9.8% (n = 1), 10% (n = 11), 12% (n = 1), 13% (n = 1), 14% (n = 1), 16% (n = 1), 17% (n = 1), 17.8% (n = 1), 20% (n = 4), 24% (n = 1), 25% (n = 2), 28.6% (n = 1), 30% (n = 1), 32% (n = 1), 34% (n = 1). These cut-off values were usually derived on the basis of median or mean values [12].

Of the many aspects influencing the evaluation of Ki67 as a prognostic or predictive marker, this study aimed to assess the distribution of Ki67 values and their relation to previously suggested cut-offs.

Materials and methods

Contributors were asked to give the Ki67 values of approximately 100 consecutive breast cancers where the staining was performed. Additional information collected in parallel included the ER, progesterone receptor (PR), and HER2 status, the age of the patient, the histological type and grade of the tumour, the mitotic score component of the combined histologic grade [13] and the specimen type on which the Ki67 values were determined. In a questionnaire, data about details of staining and evaluation were also collected. In a second round, another questionnaire specifically assessed the relation of the assessors to the recommendations of the International Ki67 in Breast Cancer Working Group (Table 1) [5]. The questionnaires can be viewed at http://bit.ly/1kxM90t. These recommendations include preanalytical details (like preferably short time to fixation, avoidance of freezing the specimen before fixation, fixation in neutral buffered formalin), the MIB-1 antibody as gold standard, the minimum number of cells to be assessed (at least 1000 suggested, and 500 stated as the absolute minimum), the handling of inhomogenous staining… etc [5] Data were collected between December 2012 and March 2013.

Table 1. Condensed summary of the recommendations by the International Ki67 in Breast Cancer working group [5] (per reviewer's request).
Preanalytical
Assessment can be done on either core needle biopsies or whole sections from resection specimens. Tissue microarrays are not recommended for quantification. Fixation should be in neutral buffered formaline with avoidance of previous freezing of the tissues (frozen sections), other fixatives and decalcination in EDTA or acid. Ideally fixation should start rapidly (especially if image analysis is planned), but delays up to 20–80 min did not abolish staining. Storing of material in paraffin embedded blocks is preferred over unstained sections on slides because antigenicity may be lost after 3 months or longer (prolonged exposure of the cut surfaces to air is to be avoided)
Analytical
Antigen retrieval is mandatory. (Microwave processing is recommended). MIB-1 antibody recommended over others. Counterstaining of all negative nuclei is important.
Interpretational
Scoring should involve the determination of the percentage of positive cells (without consideration of staining intensity) in an area with adequate nuclear staining. Considering the average score over the section is recommended (in Table 1 of the publication) [5]. In contrast, in the text, the recommendation is: Counting in at least 3 random high-power magnification (×40) fields if the staining is homogenous. Counting in 3 high-power fields at the edge of the tumour if heterogenous staining results from an increasing gradient toward the periphery of the tumourtumour (with the exception of comparative counts with previous core biopsies, where counting over the section should be performed). If hot spots are present, average scoring over the whole area is to be followed (meantime recommendation). Where scoring all cells is not practical, counting the percentage of positive cells on the basis of 1000 cells (500 cells as the absolute minimum) in an area representative of the whole section is the recommendation.
Data analysis related
No recommendation. Cut-offs should be selected on the basis of the clinical context (e.g. prognostication, response to therapy prediction… etc) and end-points should be independently validated with similar design and analogous end-points.

Results

Altogether 19 departments related to the European Working Group for Breast Screening Pathology referred data on 1782 tumours, of which 73 from one centre had to be excluded because of categorical Ki67 values (<15, 15–30, >30 per cent LI), leaving 1709 tumours for further analysis.

Methodological details are shown in Table 2. Ki67 staining was automated in 18 of the laboratories providing the results and MIB-1 antibodies were used in the majority (n = 14). The antibody dilutions varied between 1:20 and 1:500. Full tumour sections were used to establish the Ki67 LI in 72% of the cases (n = 1233). Data on 1473 tumours were the results of routine staining in consecutive breast carcinomas, whereas data on 309 tumours (from 4 departments)

were consecutive staining of non-consecutive cases (in these departments not all breast cancers were stained, but all stained cases were included). Eleven of the 19 centres/pathologists reporting data (1009 tumours) counted the Ki67 LI, whereas 8 of them used an eyeballing based estimation (773 tumours). Digital image analysis was not used for providing the presented data by any of the participating laboratories. Some method of rounding of the obtained Ki67 LI values was used in the case of 873 tumours, and no rounding of the values was made for 909 tumours. Three pathologists assessed the proportion of staining in 100 cells, 3 in 200 cells, 1 in 300 cells, 1 in 500 cells and 3 in 1000 cells. Hot spots were included by 18/19 pathologists and were the only areas assessed when present in the practice of half of them. The recommendations of the International Ki67 in Breast Cancer Working Group were known in 16 of the 19 laboratories, but were adhered to in only a minority; full adherence was found in only 3 laboratories, the deviations were mainly related to the number of cells counted or estimating rather than counting.

Table 2.
Methological details of Ki67 assessment by laboratories.

Laboratory	Antibody	Dilution	Source	Technique	Cells counted
A	MIB-1	RTU	DAKO, Glostrup, Denmark	Automated/Manual	100
B	MIB-1	1:100	DAKO, Glostrup, Denmark	Automated	NA (Estimation)
C	MIB-1	1:75	DAKO, Glostrup, Denmark	Automated	200
D	MIB-1	1:40	DAKO, Glostrup, Denmark	Automated	NA (Estimation)
E	MIB-1	1:50	DAKO, Glostrup, Denmark	Automated	200
F	MIB-1	1:50	DAKO, Glostrup, Denmark	Automated	500
G	30-9	RTU	Ventana, Tucson, AZ, USA	Automated	NA (Estimation)
H	30-9	RTU	Ventana, Tucson, AZ, USA	Automated	about 1000
I	MIB-1	1:50	DAKO, Glostrup, Denmark	Manual	1000
J	MIB-1	1:20	DAKO, Glostrup, Denmark	Automated	NA (Estimation)
K	MIB-1	1:500	DAKO, Glostrup, Denmark	Automated	100
L	MIB-1	1:200	DAKO, Glostrup, Denmark	Automated	About 1000
M	MIB-1	1:100	DAKO, Glostrup, Denmark	Automated	NA (Estimation) or 1000
N	MM1	1:100	Novocastra, Newcastle, UK	Automated	NA (Estimation)
O	SP6	1:30	Biocare, Concord, CA, USA	Automated	NA (Estimation)
P	SP6	1:400	NeoMarkers, Fremont, CA, USA	Automated	200
Q	MIB-1	1:200	DAKO, Glostrup, Denmark	Automated	300
R	MIB-1	1:125	DAKO, Glostrup, Denmark	Automated	NA (Estimation)
S	MIB-1	1:150	DAKO, Glostrup, Denmark	Automated	100

NA: not applicable.

Overall, the median Ki67 LI of the 1709 breast carcinomas analysed further was 17% with a mean ± S.D. of 23.4 ± 21% (range 0%–100%). When arranged in quartiles or terciles, the mean Ki67 LIs (%) for quartiles and terciles were 4.2 ± 2.1, 12.4 ± 2.4, 23.5 ± 4.2, 53.6 ± 18.5 and 5.6 ± 3.1, 17.4 ± 4.3, 47.3 ± 19.4, respectively. The lower half had a mean LI of 8.3 ± 4.7%, whereas the upper half was characterized by a mean of 38.6 ± 20.1%.

The Ki67 LI values were higher in cases in which the mitotic score component of the combined histological grade [13] were higher. Each score was characterised by the following respective median and mean ± S.D. Ki67 LIs: score 1: 10 and 13 ± 12; score 2: 23 and 27 ± 18; score 3: 45 and 48 ± 24. The Ki67 LIs according to grade, receptor status and molecular subtypes, as determined by the IHC surrogate approach [9], are presented in Table 3.

Table 3.

Ki67 LI values by tumor grade, receptor status and molecular subtypes as approached by IHC.

	Ki67 LI median (mean ± SD)
Histological grade	
Grade 1 (n = 386)	9 (10.6 ± 9.3)
Grade 2 (n = 834)	15 (17.7 ± 14.0)
Grade 3 (n = 489)	40 (43.2 ± 23.9)
ER-status	
ER-negative (n = 250)	50 (52.0 ± 24.8)
ER-positive (n = 1459)	15 (18.5 ± 15.8)
PR-status	
PR-negative (n = 456)	30 (37.8 ± 27.0)
PR-positive (n = 1252)	15 (18.3 ± 15.5)
PR-status unknown (n = 1)	25
HER2-status	
HER2-negative (n = 1419)	15 (22.1 ± 21.1)
HER2-positive (n = 221)	30 (33.3 ± 19.2)
HER2 inconclusive by ISH or 2 + by IHC or unknown (n = 69)	15 (19.4 ± 16.8)
IHC based molecular types [9]	
Luminal A (ER + PR + HER2-Ki67 LI < 14) (n = 622)	5 (6.5 ± 3.5)
Luminal B (ER + PR+ and HER2+ and/or Ki67 LI > 13 (n = 774)	25 (28.3 ± 15.3)
Luminal B (ER + PR + HER2-Ki67 LI > 13) (n = 626)	23 (27.7 ± 14.4)
Luminal B (ER + PR + HER2+ (n = 148)	30 (30.7 ± 18.4)
HER2 enriched (ER-HER2+) (n = 73)	31 (38.7 ± 19.7)
Triple-negative (ER-PR-HER2-) (n = 171)	60 (58.1 ± 24.3)
Not classified (n = 69)	

HER2: human epidermal growth factor receptor 2, ER: oestrogen receptor, IHC: immunohistochemistry, Ki67 LI: Ki67 labelling index, PR: progesterone receptor, +: positive, −: negative.

The Ki67 LI values showed clustering at numbers ending with 5 or 0, 1084 values (63%) clustered at zeros and fives and 653 values clustered at zeros (38%) (Fig. 1). As such clustering is to be expected with estimated values, the data were divided according to the method of evaluation (counting versus estimation) (Fig. 2) and the subset of values obtained from consecutive tumours with counting of the stained cells and no rounding of the values (n = 600) was separately analysed. Similar clustering of Ki67 LI values was present in 199 (33%) and 119 (20%) cases, respectively (Fig. 3).

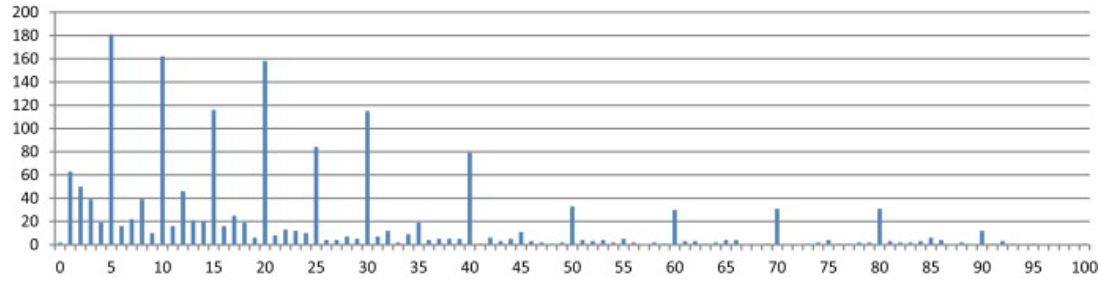

Fig. 1.

Distribution of Ki67 LI values in 1709 breast cancers (Ki67 values on axis x, absolute numbers on axis y).

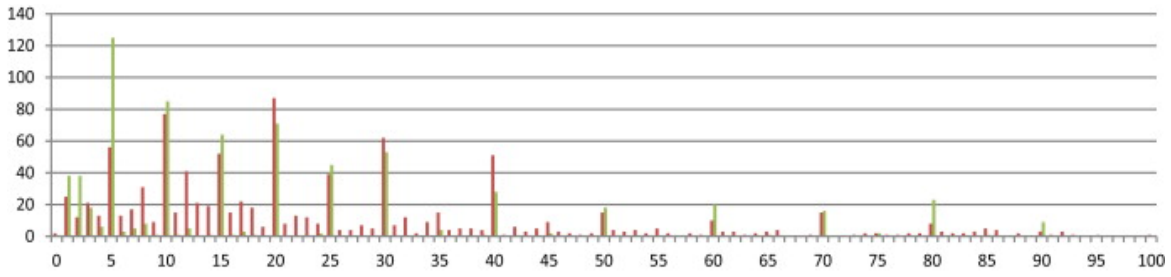

Fig. 2.

Distribution of Ki67 LI values according to the method of evaluation (Ki67 values on axis x, absolute numbers on axis y)(Counted: red (dark); estimated: green (light)). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

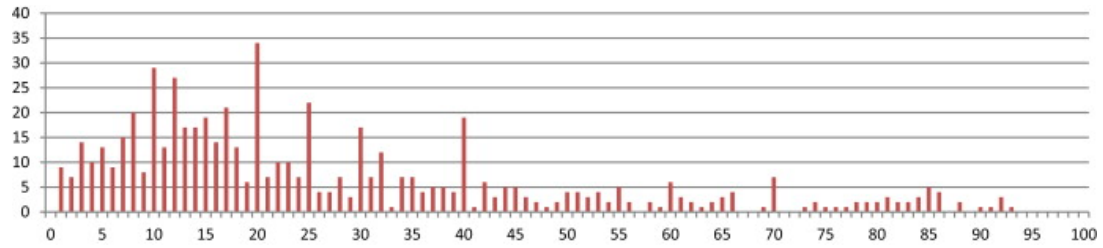

Fig. 3.

Distribution of Ki67 LI values in 600 breast cancers in which the LI was determined in a consecutive series of tumours by counting without rounding the values (Ki67 values on axis x, absolute numbers on axis y).

As Ki67 LI is often used to tailor treatment in ER-positive and HER2-negative tumours, this subset has been analysed separately. The median Ki67 LI was 14% (mean ± S.D. 17 ± 15) in the 1248 patients having this type of carcinoma. Clustering of the values was seen in both the subsets assessed by counting (n = 745) and estimation (n = 503) (Fig. 4).

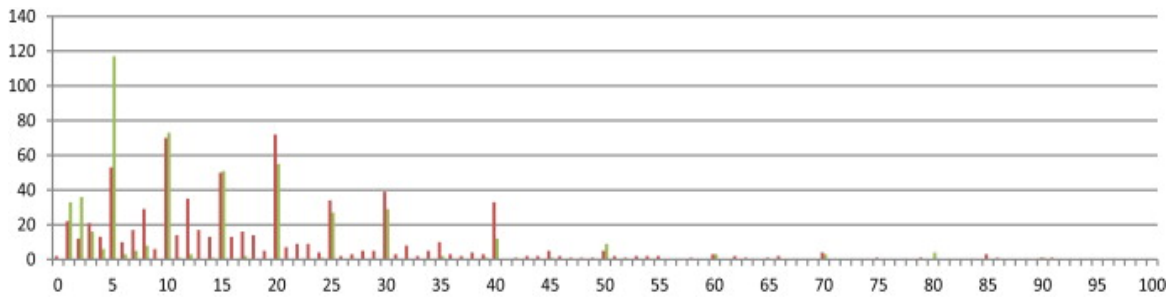

Fig. 4.

Distribution of Ki67 LI values in 1248 ER positive and HER2 negative breast cancers (Ki67 values on axis x, absolute numbers on axis y) (Counted: red (dark); estimated: green (light)). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Discussion

The data illustrated in this report clearly demonstrate that the distribution of Ki67 LI clusters at lower values (the median proportion of Ki67 estimated proliferative cells was 17% for all tumours and 14% for the ER-positive and HER2-negative subset), which reflects a tumour biology related influence on the distribution of the values, but also clusters at numbers ending with 5 or 10, which is a human evaluation related item.

Several factors influencing the determination of Ki67 LI have been mentioned in the introduction. In this series based on daily pathological methods and practices the human factor is reflected by the predilection for some LI values being more common than others in both the low and the high proliferation ranges.

Several cut-off values distinguishing between highly proliferative tumours and carcinomas with lower proliferative activity were derived from the statistical analysis of Ki67 LI values found in a given study. Both the Ki67 LI and the mitotic count are markers of cell proliferation. Although the correlation between cells positive for Ki67 and mitotic figures is obvious, and demonstrated in the present analysis too, not all Ki67-stained cells enter mitosis. Spyratos et al. have used other proliferation markers including the mitotic index to check the accuracy of Ki67 (MIB-1)-based evaluation of proliferation. By subjecting each of five different MIB-1 cut-off points to a logistic regression model, they found that the mitotic index was always the most discriminatory proliferation variable. They also found that among tumours with MIB-1 staining below 10%, 11% of tumours had a high mitotic index. Among tumours with a MIB-1 higher than 25%, 99% of

tumours had a mitotic index of 2 or 3. They concluded that with a MIB-1 cut-off point of 10%, only a few tumours with low proliferation were misclassified and the MIB-1 cut-off point of 25% adequately classified highly proliferating tumours as such [14].

As the methods of evaluation are somewhat heterogeneous and the inter-observer reproducibility of evaluating the proportion of Ki67 labelled tumour cells is less then optimal, it is not surprising that the suggested cut-offs are also variable. Any cut-off value will separate higher and lower proliferative groups of tumours, but generalization of cut-off values from a specific study [8] and [9] carries in itself a potential misclassification of some patients. Published data suggest that the reproducibility of Ki67 stained cells evaluation is not worse when the proportion is estimated rather than calculated after meticulous counting [7]. Therefore the clustering of the values illustrated on the figures of this report (which can probably be generalized) suggest that if a cut-off between highly proliferating tumours and tumours with low proliferation is to be used to allow a selection of patients at higher risk of relapse and as a factor influencing the application of systemic chemotherapy, it should consider realistic distribution of the cases, and should probably be an inclusive or non-inclusive number ending with 5 or 0 (like 10%, 15% or 20%), or more preferably ending with 0 (like 10% or 20%). For example, Reyal et al. have demonstrated the prognostic value of Ki67 in pT1-pT2 pN0 breast carcinomas with a long (over 12 years) median follow up; they have chosen 20% as cut-off [15]. Taking into consideration the distribution of Ki67 values presented in our study in comparison to previously presented cut-offs, some existing recommendations for KI67 LI in therapeutic decision making (e.g. the previous St Gallen recommendations using a cut-off of <14% for determining luminal A tumours by IHC [9]) do not seem to be reasonable. In line with our findings, while this manuscript was being continuously processed, the latest St Gallen recommendations have appeared and mention an inclusive 20% cut-off for discriminating between HER2-negative luminal B and luminal A breast carcinomas [16]. Different cut-offs may be generated for different clinical purposes.

Conflict of interest statement

None declared.

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